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Fluorescence-In-Situ Hybridization In Adult ALL
Cytogenetic Findings And Clinical Correlation

A Thesis submitted for the degree of
Doctor of Medicine of University of London

by

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Abstract

Since the first reports of cytogenetic abnormalities in ALL, repeated small and large scale studies have shown that cytogenetic abnormalities detected at presentation continue to be the most important predictor of outcome in ALL, at presentation, on relapse and even in the context of more intensive treatment modalities.

The accuracy of diagnosis is much improved by the additional application of molecular techniques including FISH, which is the technique I used to more accurately define the cytogenetic abnormalities in a series of 176 adult patients with ALL.

I have studied the incidence of and clinical features associated with three important structural abnormalities, namely *BCR/ABL*, *MLL* and *ETV6/AML1* abnormalities, as well as alterations in chromosome number and showed that FISH with commercial probes provides a simple and accurate method for the detection of these abnormalities in presentation samples. FISH detected cytogenetically silent abnormalities in twenty-four of the 176 patients. In addition to the patients positive for rearranged genes, I uncovered a rare case of Philadelphia negative *BCR/ABL* ALL with aberrant insertion of *ABL* into a morphologically normal chromosome 9q. I went on to study in detail twenty-nine cases with indeterminate cytogenetics, and was able to further characterize the karyotype in fifteen of these. I have shown the applicability of sequential FISH a technique developed in our laboratory, in identifying underlying complex chromosomal abnormalities, even when metaphase number and morphology is very poor. In a number of cases the additional findings appear to influence clinical outcome.

Finally by assessing the total cytogenetic information, as well as a number of clinical variables at presentation I developed a simple method of risk stratification for adult patients with ALL which distinguishes three subgroups, including a population accounting for 32% of 159 patients studied who appear to have significantly better outcomes when treated according to the UKALL XII protocol.

To Mohamed
Hashim & Hibbeh
And Iraq

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List of abbreviations used in this thesis

Acute lymphoblastic leukaemia (ALL)
Acute myeloid leukaemia (AML)
Argininosuccinate synthetase (ASS)
Bone marrow transplantation (BMT)
Breakpoint cluster region (bcr)
Breakpoint cluster region/Ableson (BCR/ABL)
Cancer and leukemia group B (CALB)
Central nervous system (CNS)
Chromosomal analysis (CA)
Chronic lymphoid leukaemia (CLL)
Chronic myeloid leukaemia (CML)
Cytoplasmic Immunoglobulin (cIg)
Cluster of differentiation (CD)
Common ALL (cALL)
Complete remission (CR)
4'6-diamino-2-phenyindionle dihydrochloride (DAPI)
Deletion [of chromosomal region] (del)
Derived [chromosome] (der)
Disease-free survival (DFS)
ETS variant gene 6 / Acute myeloid leukaemia 1 gene (ETV6/AML1)
Event free survival (EFS)
Extra signal (ES)
Fluorescein isothiocyanate (FITC)
Fluorescence *in situ* hybridisation (FISH)
French-American-British classification (FAB classification)
Inversion [of chromosomal region] (inv)
Kilo base (kb)
Leukaemia Research Fund (LRF)
Locus specific (LS)
Matched Unrelated Donor [for bone marrow transplant] (MUD)

Major breakpoint cluster region- (M-bcr)

Median survival (MS)

Medical Research Council (MRC)

Minimal residual disease (MRD)

Minor breakpoint cluster region- (m-bcr)

Mixed lineage leukaemia / myeloid, lymphoid leukaemia gene (MLL)

Morphologic, immunologic, and cytogenetic classification (MIC)

Multicolour / 24-colour FISH (M-FISH)

Myelocytomatosis viral oncogene (MYC)

Philadelphia chromosome (Ph chromosome)

Phosphate buffer solution (PBS)

Polymerase chain reaction (PCR)

Propidium iodide (PI)

Reverse transcriptase polymerase chain reaction (RT-PCR)

Sodium chloride, sodium citrate (SSC)

Spectral karyotyping (SKY)

Standard deviation (s.d)

Surface Immunoglobulin (sIg)

T-cell acute leukaemia 1 (*TAL-1*)

T-cell receptor alpha / beta / delta / gamma (TCRA)/ (TCRB)/(TCRD)/(TCRG)

Terminal deoxynucleotidyl transferase (TdT)

Unique identification number (UIN)

United Kingdom Acute Lymphoblastic Leukaemia trials (UKALL trials)

United Kingdom Cancer Cytogenetics Group (UKCCG)

White blood cell count (WBC)

Whole chromosome paints (wcps)

World Health Organization (WHO)

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Publications

L Harewood, H Robinson, R Harris, **M Jabbar Al-Obaidi**, G R Jalali, M Martineau, A V Moorman, N Sumption, S Richards, C Mitchell and C J Harrison; Amplification of AML1 on a duplicated chromosome 21 in acute lymphoblastic leukemia: a study of 20 cases. **Leukemia (2003) 17, 547-553 669**

M.S.J Al-Obaidi, M.Martineau, M-P Pinson, G.R.Jalali, S.A.Byatt, K.Roberts, C.F.Bennett, C.J.Harrison; *ETV6/AML1* fusion by FISH in adult acute lymphoblastic leukemia. **Leukemia (2002) 16, 669–674**

M-P Pinson, M.Martineau, **M.S.J.Al-Obaidi**, C.F.Bennet, S.A.Byatt, G.R.Jalali, K.Roberts, C.J.Harrison. Sequential FISH reveals an abnormal karyotype involving 14 chromosomes in a child with acute lymphoblastic leukaemia. **Leukaemia 2000;14(9);1705-1707**

Published Abstracts

M.S.J.Al-Obaidi, C.F.Bennet, S.A.Byatt, G.R.Jalali, M.Martineau, M-P Pinson, K.Roberts, C.J.Harrison. *ETV6/AML1* fusion in adult acute lymphoblastic leukaemia, a clinical & laboratory study. **BLOOD November 2000;96;4542a**

Jabbar Al-Obaidi M.S, Bennet C.F., Byatt S.A., Jalali G.R., Martineau M., Roberts K., Harrison C.J. Screening by Interphase FISH for high and low risk gene rearrangements in adult acute lymphoblastic leukaemia. **The Haematology Journal June 2000;1;444a**

Jabbar Al-Obaidi M.S., Bennet C.F., Byatt S.A., Jalali G.R., Martineau M., Roberts K., Harrison C.J. Sequential FISH in adult acute lymphoblastic leukaemia. **The Haematology Journal June 2000;1;448a**

M.S.Jabbar Al-Obaidi, C.F.Bennet, S.A.Byatt, G.R.Jalali, M.Martineau, M-P Pinson, K.Roberts, C.J.Harrison. Interphase FISH as a prognostic indicator in adults with acute lymphoblastic leukaemia. **British Journal of Haematology April 2000;176a**

Chapter I

Introduction

I: 1 Overview

Although a rare disease, acute lymphoblastic leukaemia (ALL) is still fatal within three years in over two thirds of adults presenting today (Wetzler 1999).

This is despite tremendous improvements in recent years in our understanding of the biology of the disease and the development of treatment protocols that offer cure rates in up to 70% of children with this disease (Hann 2000). The success story of treating children with ALL has not thus far been transported effectively to the older generation of patients.

Increasingly it is becoming recognised that individualised treatment protocols are necessary (Finiewicz and Larson 1999; Uckun 1999; Faderl 2000), which take into consideration a number of well defined prognostic factors in tailoring treatments for individual patients or patient groups with similar features. Novel methods of treatment are replacing the often-exhaustive three-year marathon of chemotherapy treatment that fails in so many adult patients.

It has been known for some time that one of the most significant predictors of outcome in ALL in both children and adults is the presence and type of chromosomal abnormalities within the leukaemic blasts at presentation, these provide a mode for prognostically classifying patients (Secker-Walker 1978; Williams 1986; Pui 1987; Crist, W. 1990; Jackson 1990; Shurtleff 1995; Chessels 1997; Heerema 1999), as well as forming the cornerstone for design of molecular studies into the pathogenesis of this most heterogeneous of malignant diseases.

Chromosomal abnormalities can also provide unique markers for the malignant cell clones allowing the identification of residual disease, as well as relapse during follow up of these patients (Secker-Walker 1989; Abshire 1992; Chucrallah 1995).

Unfortunately the routine culture of bone marrow cells from ALL patients at presentation does not always yield an informative cytogenetic result, with 8-45% of samples failing to provide any analysable metaphases (Fenaux 1989; Jackson 1990; Secker-Walker 1990; Harris 1992; Schiffer 1992; Wetzler 1999), and others showing only normal metaphases. Thus there has been increasing emphasis in recent years on molecular techniques to complement cytogenetics in the diagnostic workup of these patients, both to detect known chromosomal markers of the disease and to search for new alterations that may be associated with subgroups with specific clinical or biological features.

The aim of this project was to evaluate the molecular technique of fluorescence *in situ* hybridisation (FISH) to complement conventional cytogenetic studies for improved analysis of adult patients presenting with ALL.

I: 2 Aims of the study

I: The aim of this study was to develop a multiplex FISH protocol available for the study of all adult patients with ALL. To detect the currently known prognostically significant chromosomal abnormalities, and allow the study of individual cases in greater detail to accurately characterise ill defined karyotypes and reveal cryptic chromosomal rearrangements.

The protocol included:

1. The use of specific probes to test for abnormalities associated with poor risk disease

a. *BCR/ABL* (breakpoint cluster region/Ableson) fusion resulting from t(9;22)(q34;q11), the best-described translocation in poor outcome adult ALL.

b. *MLL* (mixed lineage leukaemia / myeloid lymphoid leukaemia) gene rearrangement, including translocations and other abnormalities involving 11q23, identifying another well-known poor risk group of patients.

2. Specific probes to test for *ETV6 (TEL)/AML1* (ETS variant gene 6 /Acute myeloid leukaemia 1 gene) fusion resulting from cryptic translocation t(12;21)(p13;q22). This recently described translocation appears to be associated with late relapse in children.

3. The use of centromere specific probes, in patients with failed cytogenetic results, or normal or ill-defined karyotypes to test for hidden numerical abnormalities. Hyperdiploidy (51-65) without t(9;22) is associated with an improved overall outcome, whereas hypodiploidy and triploidy/tetraploidy, although exceedingly rare, are associated with very poor outcome in adult ALL.

In addition to this combined approach of screening for specific gene rearrangements and numerical changes, in selected patients further FISH studies using chromosome painting probes were also undertaken to define karyotypic abnormalities and identify marker chromosomes.

II: Compare the accuracy of FISH versus cytogenetics in defining chromosomal abnormalities.

III: Evaluate accurate definition of karyotypes in terms of patients' classification into subgroups.

IV: To examine any associations between FISH /cytogenetic findings and patient outcomes.

1:3 Acute leukaemia epidemiology and aetiology

I: 3:1 Definition

Leukaemias are a group of malignancies of immature haematopoietic cells. They arise following the somatic mutation in one single haematopoietic stem cell, the progeny of which form a clone of leukaemic cells.

At the time of diagnosis, the normal marrow population has usually been completely replaced by the leukaemic clone and there has been haematogenous dissemination to a variety of extramedullary sites.

Leukaemias are classified initially on the basis of the rate of cell division and thus disease progression, into:

1 Acute leukaemia; which if untreated usually causes death in weeks or months. The diagnosis of acute leukaemia requires that blasts constitute at least 30% of either total nucleated cells or non-erythroid cells in the bone marrow. This diagnostic criterion helps distinguish the acute leukaemias from both chronic forms of leukaemia and most lymphomas.

2 Chronic leukaemia; which even without treatment may be asymptomatic for several months or years, and causes death in months or years.

I: 3:2 Aetiology of leukaemia

Despite extensive research the aetiology of leukaemia is still largely unknown (Sandler 1992). There have been dramatic changes over time in leukaemia mortality and incidence. Mortality from leukaemia increased substantially between the 1920s and about 1960 (Sandler 1992; Miller 1993), probably due to the combined effect of the reduced incidences and improved survival rates of other diseases, improvements in diagnostic accuracy of leukaemia, and possibly the effects of some new environmental hazards. Since the mid-1970s, there has been an overall slight decrease in leukaemia incidence (Miller 1993).

A number of environmental agents have been implicated in the induction of certain types of leukaemia. These include ionising radiation, chemical carcinogens (mainly alkylating agents used to treat other malignancies) and benzene (Sandler and Collman 1987). In addition, acute leukemia may develop due to host susceptibility in some genetic disorders including Down's syndrome and ataxia telangiectasia (Pui 1995; Takeuchi 1998). There is evidence that certain polymorphisms in methyltetrahydrofolate reductase [a gene involved in folate metabolism] may modulate the risk of developing ALL (Skibola 2002). Studies in twin have shown that childhood acute lymphoblastic leukaemia is frequently initiated by a chromosomal translocation event in utero followed by a postnatal promoter event (Weimels 1999). Acute leukaemias may also supervene as blast transformation in pre-existing myeloproliferative disorders.

A/ Radiation

X-rays and other ionising rays were first identified as agents associated with the induction of leukaemia in the early cohorts of radiologists, and the survivors of the atomic bomb explosions in Hiroshima and Nagasaki. People exposed at a younger age or closer to the hypocentre of the bomb had shorter latent periods for the development of leukaemia. Acute myeloid leukaemia (AML) and chronic myeloid leukaemia (CML) were the predominant types although cases of ALL were also reported. There are abundant case reports of patients treated with irradiation for other malignancies mainly Hodgkin's disease and carcinoma of the breast, or benign conditions such as ankylosing spondylitis, developing leukaemia, mainly AML, but an increase in all leukaemia types except chronic lymphoid leukaemia (CLL) are generally reported following radiation exposure (Inskip 1993). Evidence for a leukaemogenic effect of low dose irradiation e.g. exposure to diagnostic X-rays or radioisotopes at diagnostic levels has not been substantiated except in infants whose mothers were exposed to X-rays during pregnancy (Sandler 1992).

A number of surveys in the UK have suggested a clustering of cases of childhood ALL in the vicinity of nuclear installations, these studies are difficult to interpret because of the small numbers of cases expected in these populations (Sandler 1992) and other studies demonstrated no increase in leukaemia incidence among nuclear workers (Jablon and Boice 1993).

The role of non-ionising radiation especially electro-magnetic fields is also under debate. Not all studies in cohorts of workers exposed to electro-magnetic fields have demonstrated increased risk (Bates 1991; Sahl 1993). These studies also failed to

isolate electro-magnetic fields as the causal agents, because the workers were often also exposed to solvents and other agents. Studies of residential exposure have suggested a possible leukaemia risk associated with electro-magnetic fields (Bates 1991; Savitz 1993).

B/ Chemicals

Two types of chemicals are involved in leukamogenesis, benzene and other petroleum derivatives, and chemotherapeutic agents.

Benzene related haematological changes were described as early as 1897, and the first case of benzene-associated leukaemia was reported in 1928 (Austin 1988). Most reports of haematological changes due to benzene come from groups exposed well before the current occupational standards, and there have been reports of leukaemia in workers first hired in the mid 1970s. In an increasing number of cases alkylating agents have been directly implicated in the induction of secondary AML, the resulting leukaemia sometimes evolved slowly with myelodysplastic changes and a significantly high incidence of chromosomal abnormalities, particularly involving chromosomes 5 and 7. The cumulative dose of alkylating agents and patient age are the main determinants for developing AML, the risk increases from one year after the start of treatment and rises steeply in the first 1-2 years levelling off at 7 years. The nitrosureas and epipodophylotoxins have been associated with subsequent AML after a relatively short latency period of 1-5 years. They often have cytogenetic abnormalities involving chromosome band 11q23 and the *MLL* gene (Secker-Walker 1998).

In addition there have been at least ten published studies on smoking and leukaemia.

Almost all have demonstrated small but statistically significant increase in ALL risk in smokers older than 60 years (Sandler 1993).

I: 3:3 Epidemiology of acute lymphoblastic leukaemia

There are no major differences in incidence according to geographical area or between urban industrialised and rural areas. Some sociocultural factors appear to influence the incidence of CD10 (cluster of differentiation 10) positive form of ALL known as common ALL (cALL), which is less frequent than T-ALL in African countries and in poorer sections of the community in the United States of America e.g. blacks or Spanish (Hoelzer and Gokbuget 2000).

The highest incidence of ALL is seen in children aged 2-5 years, with a modest secondary increase in frequency beginning at about 50 years of age. The annual incidence in children 15 years or younger in the United States is reported as 3 cases per 100,000 for whites, but no more than 1.4 cases per 100,000 for blacks (Young 1986). Between 1984 and 1986 ALL occurred in the United Kingdom at ages 0-84 years at an overall rate of 1.3 per 100,000, with a peak incidence between 0-4 years of age at 5.1 per 100,000, diminishing thereafter and becoming rare between the ages of 25 and 44 with a slight rise between the age of 75-80 (1.8 per 100,000) (McKinney 1989).

More males than females present with ALL, the sex ratio at all ages is 1.4:1.0 (McKinney 1989).

I: 4 Methods of Classifying Acute Leukaemia

Acute leukaemia comprises a heterogeneous group of conditions differing in aetiology, pathogenesis, natural history, and prognosis. The classification into subgroups enables an improved understanding of the leukaemic process and the increased likelihood of causative factors being recognised.

The precursor cell in which the leukemic transformation occurs may be lymphoid, or myeloid in origin or a cell that is capable of differentiating into both lymphoid and myeloid lineages. Based on the origin of the cells with leukaemic transformation, leukaemia can be classified into lymphoid and myeloid types.

It is also customary to divide patients with acute leukaemia into two main age groups; childhood acute leukaemia (<15 years) and adult acute leukaemia (>15 years). The main difference between childhood and adult disease is that ALL constitutes over 80% of childhood cases, whilst AML constitutes around 80% of adult cases, some other age subgroups are often considered separately, adults aged >60 years, because their response to treatment protocols for acute leukaemia is inferior and because these patients are often not included in the more radical approaches using autologous or allogeneic transplantation, adolescents or young adults aged 15-21 as a subgroup may be important in ALL as it lies biologically between childhood and adult ALL. These patients tend however to have better responses when treated according to protocols akin to those used for high risk childhood disease (Nachman 1993). ALL in infants (less than 1 year of age) is also increasingly being treated according to protocols that are more intensive but shorter in duration than those for older children. (Isoyama 2002)

The ideal classification of acute leukaemia must be biologically relevant, reproducible, and widely applicable. Rapid categorization should be possible so that therapeutic decisions can be based on the results of the classification. A method of classification that does not change over time also allows comparisons to be made between different groups of patients or different treatment modalities.

There are currently three important methods of classifying the acute leukaemias, morphology, immunophenotype, and cytogenetics.

I: 4:1 Morphological classification

The first step in classifying an acute leukaemia is the division of cases into acute myeloid leukaemia, and acute lymphoblastic leukaemia.

The importance of this first step of categorization is paramount, since the natural history differs considerably between the two conditions and the modes of treatment are sufficiently different for an incorrect diagnosis to adversely affect prognosis.

The first step in distinguishing these two types is based on the morphology of the cells.

The morphologic classification most commonly employed is the French-American-British (FAB) (Bennett 1976), which is dependent on morphological features of blast cells when stained with a Romanowsky and cytochemical stains. The FAB classification allows the morphological recognition of most cases of AML from the presence of myeloperoxidase positive Auer rods, or monocyte-associated esterases in blast cells, with the exception of AML subtype M0 with negative cytochemistry by light microscopy.

The FAB classification classifies lymphoid blasts into three subtypes:

L1 morphology:

The blasts are small and uniform, with scanty moderately basophilic cytoplasm; nuclei are regular in shape with inconspicuous nucleoli.

L2 morphology:

The blasts are large and non-uniform, with variable amounts of basophilic cytoplasm; nuclei are irregular in shape with prominent nucleoli.

L3 morphology:

The blasts are large and uniform; with moderately abundant amounts of deeply basophilic cytoplasm with prominent vacuoles nuclei are regular in shape with prominent nucleoli.

In childhood ALL, the distribution of cases between L1, L2 and L3 is 85%: 14%: 1%.

Most adult patients have L2 type with L1: L2: L3 ratio of 31%: 60%: 9%.

Morphologic or cytochemical features in ALL are more subjective than in AML and have not been on the whole useful in subdividing ALL, with the exception of the rare L3 or Burkitt subtype of ALL. This type lends itself well to morphological identification and, as patients with this disease require different treatment to others, morphological examination remains of importance to detect these rare cases.

However in the remaining >90% of cases the contemporary diagnostic work-up of ALL relies most heavily on immunophenotyping and cytogenetics.

I: 4:2 Immunophenotypic classification

The ability to classify ALL by lineage commitment and degree of maturation features displayed by the leukaemic blasts (immunophenotyping) began in 1975 (Greaves 1975; Sen and Borella 1975), the blasts are classified according to the presence or absence of specific nuclear or surface markers that identify them as being arrested at a certain stage in B or T lymphocyte development. The leukaemia is classified according to this cell type and nomenclature is based on the closest known normal B or T cell progenitor.

Characterisation of leukaemic blasts by immunophenotyping is now usually undertaken with a flow cytometer and a panel of specific monoclonal antibodies that identify cell surface antigens and cell markers with a specific CD designation (Foon 1990).

The development of new fluorochrome reagents for cell labelling and sensitive, reliable flow cytometers, capable of measuring several different cellular properties simultaneously, has greatly improved the precision of the procedure (Greaves 1981). The list of leukocyte differentiation antigens and the monoclonal antibodies that recognize them has grown rapidly. At the 7th International Workshop on Leukocyte Differentiation Antigens (Zola 2003), 246 CD groups were designated, but only a few markers have lineage specificity. By this mode of classification lymphoid blast cell types fall broadly into two categories: B cells and T cells. (Table 1.1)

The earliest indication of lineage commitment is rearrangement of immunoglobulin genes of B-cell leukaemia, with expression of cytoplasmic followed by surface immunoglobulin heavy chain (Korsmeyer 1983; Ha 1985), and antigen receptor

genes of T-cell leukemia (Tawa 1985).

Testing with a variety of monoclonal antibodies specific for antigens is used to define the level of maturation achieved by the blasts from the most primitive or most aberrant pre-pre-B or null cells to mature B cells as shown in Table 1.1. B-lineage ALL accounts for 75-80% of cases, with cALL being the most common in childhood (60-70%), while pro-B ALL is rare in children, but occurs more frequently in adults (20-40%).

T-ALL comprises 13-20% of childhood ALL cases and up to 25% in adults (Foa 1985; Kalwinsky 1985). T-ALL blasts are subdivided according to maturity into pre-T, intermediate, late and mature T subtypes as presented in Table 1.2.

Immunophenotypic analysis is critical to confirm a morphological diagnosis of ALL, and to sub-classify cases into precursor-B and precursor-T lineage types. However, the distinctions between and the terminology for the different subtypes of these two main groups are not universally agreed upon. In fact due to the lack of conformity and the questionable significance of further sub-classification, the World Health Organisation (WHO) classification scheme which incorporates cytology, histology and genetic abnormalities in the classification of haematopoietic malignancies simply classifies cases as precursor-B and precursor-T ALL without additional categorization, and classifies the Burkitt's Leukaemia and Burkitt's lymphoma together as mature B-cell neoplasia (Jaffe 2001). For purposes of treatment planning, the only critical distinction is between mature B-cell and B-cell precursor ALL. On the other hand a specific immunophenotype identified at diagnosis might be useful for evaluating residual disease by flow cytometry after treatment.

Table I.1 Immunophenotype in B-ALL

Normal counterpart	Leukaemic blast type	CD19	CD10	cIg	sIg
Pre-pre B/Pro-B	Pro-B/Null	+	-	-	-
Early pre-B	c-ALL	+	+	-	-
Late pre-B cell	Pre-B ALL	+	+/-	+	-
Mature B cell	B-cell ALL (Burkitt type)	+	-	+	+
cIg =Cytoplasmic Immunoglobulin		sIg= surface Immunoglobulin			

Table I.2 Immunophenotype in T-ALL

Normal counterpart	Leukaemic blast type	CD7	CD2	CD4	cCD3	CD8	TCR $\alpha\beta$
Bone marrow Prothymocyte	Pre-T	+	-	-	-	-	-
Thymic cortex thymocyte	Intermediate T	+	+	+	+	+	-
Thymic medulla thymocyte	Late T	+	+	+/-	+	-/+	+
Peripheral blood T cell	Mature T	+	+	+/-	+	-/+	+
cCd3=cytoplasmic CD3		TCR=T-cell receptor gene					

I: 4:3 Cytogenetic classification

Leukaemia arises from a hematopoietic progenitor cell that has sustained specific genetic damage, leading to malignant transformation and growth. Thus, genetic classification of ALL should yield more useful information than that obtained with other systems.

Classification of patients with ALL by chromosomal abnormalities was originally based on counting the chromosomes and arranging them into seven categories, A-G. This form of classification was shown to be prognostically important in 1978, when it was found that hyperdiploidy (>50 chromosomes) in childhood ALL carried a better prognosis (Secker-Walker 1978). Later it was shown that the presence of an abnormally short chromosome 22 called the Philadelphia (Ph) chromosome, which resulted from the translocation $t(9;22)(q34;q11)$ was associated with a poor outcome in ALL (Secker-Walker 1982). With improved preparation and chromosome banding techniques it became possible to classify leukaemic blasts according to chromosomal abnormalities. Currently by standard karyotyping, more than 80% of ALL cases show clonal chromosomal abnormalities, half of which are translocations (Secker-Walker 1990; Schiffer 1992; Secker-Walker 1997; Harrison and Foroni 2002).

The cytogenetic analysis of leukaemic blast cells has become an indispensable tool in diagnosis and risk assessment of ALL, and can be used to pinpoint chromosomal sites for the molecular cloning of proto-oncogenes.

A: Numerical chromosomal changes

Cytogenetic changes are classified according to number of chromosomes:

Near haploid; 23-29 chromosomes

Low hypodiploid; 30-39 chromosomes

High hypodiploid; 40-45 chromosomes

Normal; 46 chromosomes, without structural abnormalities

Pseudodiploid; 46 chromosomes, with either structural abnormalities, or gains of chromosomes compensated by losses of other chromosomes

Low hyperdiploidy; 47-50 chromosomes

High hyperdiploid; 51-65 chromosomes

Near triploid; 66-80 chromosomes

Near tetraploid with >85 Chromosomes

Numerical chromosomal changes were the first cytogenetic anomaly to be described in leukaemia. And to date, after the description of numerous structural abnormalities, the presence of an abnormal chromosome count remains an important classification tool in ALL.

Losses of chromosomes are generally associated with deteriorating outcome, near haploid karyotypes are rare in both adults and children, with an incidence of less than 3%, and are associated with median survival of 11 months (Gibbons 1991; Secker-Walker 1997; Heerema 1999; Harrison 2004). Poor outcome is also associated with hypodiploidy with 44 chromosomes or less in childhood ALL (Heerema 1999), and with loss of chromosome 7 in adults (Wetzler 1999).

In contrast high hyperdiploidy (modal numbers of 50-68 chromosomes) is associated with good risk ALL, a disease characterized by specific age groups, two to nine year olds in children, and below 18 years in adults, leukocyte counts of $<10 \times 10^9/l$, female sex, and c-ALL immunophenotype (Walters 1990; Secker-Walker 1997). In children, where high hyperdiploidy occurs in over one third of cases it is associated with 5-year survival rates of 72%-80% (Pui 1989; Jackson 1990; van der Plas 1992; Raimondi 1996; Heerema 2000). In adults high hyperdiploidy accounts for

approximately 10% of cases. When not associated with other structural abnormalities, such as translocation t(9;22), it predicts a better outcome with three-year event free survival (EFS) rates of 59% (Secker-Walker 1997).

Within hyperdiploid clones chromosomal gains are restricted to certain chromosomes, in low hyperdiploidy the chromosomes most frequently acquired are chromosomes 21 (42%), X (22%), 10 (13%), 8 (11%), 6 (9%) and 16 in (7%), in high hyperdiploidy those most frequently gained are chromosomes 21 (100%), X (95%), 6 (86%), 18 (78%), 4 (75%), 17 (65%), and 10 in (61%) (Moorman 1996).

Within the ploidy groups, gain or loss of specific chromosomes plays an important role in predicting outcome. Early studies found the combination of trisomies for chromosomes 4 and 10 in high hyperdiploidy predicted the best prognosis in children (Harris 1992), more recently the presence of trisomies for chromosomes 4 and 18 in high hyperdiploidy has been found to predict five year survival of 96% (Moorman 2003). Whereas trisomy 5 confers a poorer outcome within this group of patients (Heerema 2000). Trisomy 8 has been associated with poor outcome in adults (Wetzler 1999).

Numerical changes can be associated with structural changes including t(4;11)(q21;q23), t(8;14)(q23;q32), and t(9;22)(q34;q11). One fifth of adults with Philadelphia chromosome positive ALL have hyperdiploid clones (Crist, W. 1990; Secker-Walker 1997). The influence of co-existing structural abnormalities remains unclear. Childhood ALL studies show conflicting results: Van der Plas et al showed better prognosis in the absence of structural abnormalities (van der Plas 1992), Raimondi showed the reverse (Raimondi 1996). In adults from Medical Research

Council United Kingdom Acute Lymphoblastic Xa trial (MRC UKALLXa) the better outcome with high hyperdiploidy was only seen in those patients without structural abnormalities (Secker-Walker 1997).

The effect of low hyperdiploidy (47-50) chromosomes which occurs in 11% of children and adults (Fletcher 1989; van der Plas 1992; Harbott 1993; Raimondi 1993; Kobayashi 1994) is less clear, but appears to predict slightly better outcomes in adults (Secker-Walker 1997).

B: Structural chromosomal abnormalities:

B-precursor ALL

Non-random chromosomal rearrangements are common in ALL: Table 1:3 lists the most common. Some of these have clinical significance, including:

i: The translocation t(9;22)(q34;q11), giving rise to the Philadelphia chromosome. The Ph chromosome is the cytogenetic hallmark of CML (Nowell 1960), later described in childhood (van Biervliet 1975) and adult ALL (Propp and Lizzi 1970). It occurs in 5% of children and 20-35% of adults with ALL (Ribeiro 1987; Secker-Walker 1991; Westbrook 1992). ALL patients carrying the Ph translocation present with white blood cell counts (WBC) which range from 3 to $>100 \times 10^9/l$. Higher WBC are found in childhood Ph positive ALL than in other childhood patients (Crist, W. 1990). This difference is not consistently seen in adults (Secker-Walker 1991).

The incidence of Ph positive ALL increases with age from approximately 2% in children <15 years of age (Secker-Walker 1990) to 10% at 15-20 years, 19% at

21-30 years, 24% at 31-50 years, and up to 44% in those over 50 years old at the time of presentation (Secker-Walker 1991).

Blast cell immunophenotype shows that the vast majority of Ph positive cases have B-lineage blasts. In children, 74% show cALL, a further 16% have a pre-B phenotype and only 10% have blasts with positive markers for T-ALL (Ribeiro 1987; Crist, W. 1990). Similarly in adult series, the overwhelming majority of Ph positive ALL show either cALL or pre-B phenotypes (Secker-Walker 1991; Secker-Walker 1997).

Patients with Ph positive ALL, particularly adults, have a dismal prognosis when treated with standard chemotherapy, and are treated according to high-risk treatment protocols, (Bloomfield 1986; Maurer 1991; Secker-Walker 1991; Gotz 1992; Westbrook 1992; Rieder 1993; Annino 1994; Secker-Walker 1997).

More recently, Imatinib (Gleevec, STI-571), a relatively specific BCR-ABL tyrosine kinase inhibitor, has shown marked therapeutic benefit in CML (Druker 2001) and in the short term, at least, in Ph positive ALL (Ottmann and Hoelzer 2002; Wassmann 2002; Wassmann 2003). It has been incorporated into treatment regimens for this subgroup of patients, including patients entered to the UKALL XII trial, and the new childhood ALL trial ALL2003.

However even within this group of patients there seems to be some heterogeneity, for example the additional finding of monosomy 7 or abnormalities involving the short arm of chromosome 9 in Ph positive ALL are associated with low remission rates of extremely short duration (Rieder 1991; Russo 1991; Rieder 1996; Heerema 2004). In contrast the finding of hyperdiploidy in Ph positive disease is associated

with higher rates of remission and an overall improvement in survival (Rieder 1996).

ii: 11q23 abnormalities

A second group of significant chromosomal abnormalities are structural rearrangements of chromosome 11 at band q23. Rearrangements include deletions, duplications, inversions and reciprocal translocations (Secker-Walker 1998). Most of these abnormalities involve the *MLL* gene at 11q23. As a group they account for 5-10% of acquired chromosomal rearrangements in children and adults with ALL, AML, poorly differentiated or biphenotypic leukaemias and myelodysplastic syndromes (Mitelman 2004). They occur in 70% of acute leukaemias in neonates and up to 85% of secondary leukemia resulting from exposure to topoisomerase II inhibitors such as the epipodophyllotoxins. Most of these are AML (Reaman 1985; Chen 1993; Rubnitz 1994; Secker-Walker 1998; Rowley and Olney 2002), but there have been approximately 50 cases of therapy related ALL reported (Andersen 2001). The largest series of 11q23 abnormalities was reviewed by the European Union (EU) Concerted Action Workshop on 11q23 Abnormalities in 1998. This showed that the majority of patients exhibited reciprocal translocations joining 11q23 with as many as 30 cytogenetically distinguishable partner loci. The most common partners were found to be 4q21 in t(4;11)(q21;q23) translocation, and 9p21~22 in t(9;11) accounting for 40% and 27% of translocations respectively (Johansson 1998; Swansbury 1998). Another frequently encountered rearrangement was t(11;19) involving the p13 band of chromosome 19 which accounted for 12% of the translocations (Moorman 1998). Additions, duplications, and inversions accounted for 16% of 11q23 abnormalities (Harrison 1998).

The translocation, t(4;11)(q21;q23), is specifically associated with a Null cell immunophenotype (Pui 1992). Blasts may also have myelomonocytic characteristics (Hagemeyer 1987). Another feature is their association with a WBC > 100x10⁹/l, seen particularly in infants and older patients.

The adverse prognostic significance of 11q23 rearrangements has been noted for some time (Raimondi 1989; Rubnitz 1997; Pui 2003). Patients tend to enter remission with the same frequency, but relapse more frequently and have shorter remission durations than patients without 11q23 abnormalities. Children with B-precursor ALL and t(4;11) t(9;11), or t(11;19) have 5 year EFS of 11% compared with 74% for patients without 11q23 abnormalities (Pui 1991; Rubnitz 1997). Pui stratified paediatric patients with t(4;11) positive ALL by age and found significantly shorter EFS in children under 1 year of age and over 10 years old, but not for children between the ages of 1 and 10. Taking all age groups represented in the EU 11q23 Workshop, a similar difference was found, with 3-year EFS rates of almost 75% in the 2-9 year olds, dropping to 40% in 10-39 years old, and those less than 2 years of age. The worst outcome was seen in patients over 40 years of age, in whom the 3-year EFS was only 14%. There is some evidence that overall survival (OS) can be improved in these patients by more intense therapy, including allogeneic transplantation in first remission (Ludwig 1998). Other abnormalities of 11q23 in ALL have different outcome, for example it seems that outcome is related to *MLL* involvement rather than cytogenetic evidence of 11q23 deletions in ALL (Harbott 1998).

iii: t(1;19)(q23;p13)

Another translocation in pre-B ALL is the t(1;19)(q23;p13), of which there are two forms a balanced t(1;19)(q23;p13) and an unbalanced form der(19)t(1;19). The translocation was first described in 1984 (Carroll 1984; Michael 1984), occurring in 2-9% of children with ALL (GFCH 1993; Harbott 1993; Pui 1994; Secker-Walker 1997) and 2% of adults (GFCH 1996; Secker-Walker 1997). Blasts express cytoplasmic immunoglobulin in a high percentage of cells (Carroll 1984). The prognostic implications of this abnormality are not entirely clear. Initially it was identified as a major adverse feature in pre-B ALL (Crist, W. M. 1990). Further studies from St Jude's hospital showed that the more intensive treatments, now used as standard therapy improved the survival rates of children with pre-B ALL with any translocation and t(1;19) in particular (Raimondi 1990). In one large study it was shown that the unbalanced form was associated with a better prognosis than the balanced form (Secker-Walker 1992). The small number of adults with either form treated within the MRC UKALL Xa trial (1985-1992), had an EFS of 54% at three years, and not statistically significantly different from the group as a whole.

iv: t(12;21)

The most common structural cytogenetic abnormality found in children with ALL is the t(12;21)(p13;q22) involving the *TEL-AML1(ETV6-RUNX1)*. This translocation is invisible by conventional cytogenetic analysis, because the rearrangement does not alter the banding pattern of the involved chromosomes. When analysed by FISH or polymerase chain reaction (PCR) the t(12;21) is found in 16-32% of paediatric B-lineage ALL cases but in only 3-7% of adult cases (Faderl 1998; Harrison 2001;

Jabbar Al-Obaidi 2002). Clinically the majority of such patients present with a precursor-B-phenotype at 3-6 years of age. These patients have an excellent prognosis with EFS approaching 90% with the best results obtained with regimens using intensive L-asparaginase treatment (Borkhardt 1997; Rubnitz 1997). Although late relapses some occurring after more than 10 years have been reported (Harbott 1997), it is possible that in some of these cases the TEL / AML clone persists in long term remission (Endo 2003), and that subsequent relapses occurs due to the acquisition of further genetic alteration such as deletion of the second TEL (Zuna 2004).

Mature B-cell ALL

B-cell leukaemia is found in 1% to 3% of childhood and adult ALL, usually characterized by FAB L3 cellular morphology (Bennett 1976), the presence of monoclonal Ig on the surface of malignant cells (Flandrin 1975) and extramedullary diseases (Patte 1986; Sullivan 1990).

Over 80% of mature B-cell ALL have one or other of the three translocations consistently found in Burkitt's lymphoma t(8;14)(q24;q32) (80%), t(2;8)(p12;q24) (6%) or t(8;22)(q24;q11) (14%) (Kornblau 1991). Although Burkitt lymphoma associated with 8q24 translocations were once associated with a poor prognosis, multiple studies now show improved outcome in both children and adults with the use of short term chemotherapy, using high doses of an antimetabolite (methotrexate) and alkylating agents (cyclophosphamide, ifosfamide) (Hoelzer 1996; Lee 2001).

T-cell ALL

In T-cell ALL, the frequency of chromosomal rearrangement is low at 44%. This may be a reflection of small abnormal clones that are difficult to detect, or the presence of cryptic abnormalities. The most common abnormalities involve the *TAL-1* (T-cell acute leukaemia 1) gene on chromosome 1p32. Other common abnormalities involve breakpoints at 14q11, 7q32 or 7p15 (Raimondi 1988), the sites of the T-cell receptor alpha/delta [*TCRA/TCRD*], beta (*TCRB*) and gamma (*TCRG*) genes respectively. The recently described recurrent cryptic translocation t(5;14)(q35;q32), which is seen in 16-22% of childhood and adolescent T-ALL, results in overexpression of the *HOX11L2* gene (Bernard 2001). There is no apparent relationship between particular translocations and the stage of thymocyte differentiation of leukaemic blasts. In general chromosomal abnormalities have been found to correlate with a poor outcome in T-cell ALL (Pui 1990) but insufficient cases have been studied to determine the unique impact of specific rearrangements.

There are some important associations among the three methods of classifying the acute leukaemias, which prompted the integration of morphology with immunology and karyotype of the neoplastic cells. This classification became known as the MIC (morphologic, immunologic, and cytogenetic) classification 1986 (MIC 1986) as summarised in Table 1.4.

Of note is that the L3 morphology is consistently associated with the B-cell leukaemia immunophenotype, surface immunoglobulin and the t(8;14) translocation or one of its variants.

The t(4;11)(q21;q23) translocation is frequently associated with blast cells of mixed lymphoid and myeloid antigenic markers and associates with pro-B ALL, the t(1;19)(q23;p13) and t(12;21)(p13;q22) associate with pre-B ALL,. The t(9;22)(q34;q11) blasts are more usually cALL. The t(11;14)(p13;q11) associates with T-cell ALL. Hyperdiploidy is more common in children with early pre-B ALL, translocations are less common in this group. Pre-B ALL is less likely to include hyperdiploidy and translocations are a more frequent finding.

The L1 or L2 morphology has no consistent correlation.

Table 1.3 Recurrent chromosomal abnormalities in ALL

			Frequency % in ALL		DFS at 3-5yr	
			Children	Adults	Children	Adults
8q24 translocations	Mature B	<i>MYC,IGH/IGK/IGL</i>	2	3-5	75-85	60
t(4;11)(q21;q23)	Pro-B	<i>MLL,AF4</i>	70 (infants)	3-6	9mo (MS)	7 mo (MS)
t(1;19)(q23;p13)	Pre-B	<i>E2A,PBX1</i>	5-6	1-3	70-80	10-50
t(9;22)(q34;q11)	B-lineage	<i>BCR,ABL</i>	3-4	25-30	20-40	<10
t(12;21)(p13;q22)	B-lineage	<i>ETV6,AML1</i>	20-25	1-3	85-90	10-50
14q11, 7q35, 7p14-15	T-lineage	TCR rearrangements	10	<5	65-75	60
del 9p	B or T	p16 ^{INK4A} ,p14 ^{ARF} ,p15 ^{INK4B}	?	12	<10	40
del 6p	B or T		32	3	>70	?
<46 Chromosomes	B or T		7	4-5	25-40	10
51-65 Chromosomes	B or T		25	2-5	80-90	40-50

DFS=disease free survival

mo=months

MS=median survival

TCR=T-cell receptor

Table I.4 The MIC classification of ALL

Disease category	Morphology	Immunology	Karyotype
FAB			
B-lineage ALL			
Early B-precursor ALL	L1,L2	TdT+,CD19+,CD10-	t(4;11), t(9;22)
Common ALL	L1,L2	TdT+,CD19+,CD10+	6q-,near haploid,t or del(12p), t(9;22)
Pre-B ALL	L1	TdT+,CD19+,Cylg	t(1;19), t(9;22)
B-cell ALL	L3	TdT-,CD19+,Smlg	t(8;14), t(2;8), t(8;22),
T-lineage ALL			
Early T-precursor ALL	L1,L2	TdT+,CD2-, CD7+	t or del(9p)
T-cell ALL	L1,L2	TdT+, CD2+, CD7+	t(11;14), 6q-

TdT= Terminal deoxynucleotidyl transferase

I: 5 Molecular Genetic Events in the Pathogenesis of ALL

The observation that chromosomal gains or losses as well as structural abnormalities can identify subgroups of ALL that differ in their response to chemotherapy prompted several studies to identify biological explanations for these differences. The favourable prognosis of hyperdiploidy has been attributed to the low leukaemic cell burden associated with this cytogenetic feature, a tendency to accumulate an increased amount of methotrexate polyglutamates, an increased sensitivity to antimetabolites, and a marked propensity for spontaneous apoptosis (Belkov 1999; Ito 1999). For the most part, biological explanations for the prognostic impact of other genetic abnormalities remain uncertain.

There are two mechanisms by which chromosomal translocations contribute to ALL pathogenesis

1:5:1 Chimeric proteins

The best-studied examples of chromosomal translocation leading to chimeric protein formation are:

i: $t(9;22)(q34;q11)$ *BCR/ABL*

This translocation results from the fusion of the *BCR* gene at 22q11 to the *ABL* gene at 9q34. This results in the generation of a protein composed of the 5' part of the *BCR* and the 3' part of the *ABL*. Fusion of *BCR* to *ABL* results in activation of *ABL*, a non-receptor tyrosine kinase whose activity is normally very tightly regulated (Davis 1985).

The sites of breakpoint within *ABL* are variable within a 300 kilo base (kb) segment of intron 1 at the 5' end of the gene, in *BCR* the breakpoints are more restricted.

In CML and some cases of ALL the *BCR* breakpoint is localized to a 5.8 kb region – the major breakpoint cluster region- (M-bcr) spanning 5 exons b1 to b5, usually resulting in a hybrid *BCR-ABL* mRNA molecule with a b3a2 and/or b2a2 junction, encoding a new protein of 210 kilo daltons. In 90% of childhood ALL and 50% of adult ALL the *BCR* breakpoint is 5' to M-bcr, within a 200kb span referred to as the –minor breakpoint cluster region- (m-bcr) between two e2' and e2 exons, the resultant mRNA codes for a 185 to 190 kilo dalton fusion protein.

The precise mechanism by which BCR-ABL chimeric protein transforms cells remains elusive. What is known is that it can transform haematopoietic cells so that their growth and survival in vitro becomes independent of cytokines (Gishizky and

Witte 1992). The BCR-ABL chimeric protein can also protect haemopoietic cells from apoptosis in response to cytokine withdrawal and DNA damage by chemotherapy and radiotherapy (Evans 1993; Nishii 1996). Biochemical studies of leukemogenesis indicate that the BCR-ABL protein is a constitutively active tyrosine kinase confined to the cytoplasm, whereas the wild type ABL shuttles between the nucleus and cytoplasm (Van Etten 1989). As a consequence of increased tyrosine kinase activity, the BCR-ABL protein can phosphorylate several substrates, thereby activating multiple signal transduction cascades, affecting the growth and differentiation of cells.

ii: 11q23 *MLL* translocations

Breakpoints at 11q23 disrupt the 100 kb *MLL* gene between exons 5 and 11 (Djabali 1992; Tkachuk 1992). *MLL* has partial homology to the *Drosophila* transcription factor trithorax, which is thought to regulate a number of homeotic genes in the fly. It remains unknown how dysregulated *MLL* function contributes to leukomogenesis in humans.

Reciprocal translocations result in the replacement of *MLL* coding sequences 3' to the breakpoint by the translocation partner. The derivative chromosome 11, which is universally transcribed by the leukaemic clone (Rowley 1992; Johansson 1998) retains the *MLL* promoter as well as amino terminal *MLL* coding sequences up to exon 5-11, depending on the exact breakpoint.

MLL is among the most promiscuous oncogenes in human leukemia, with over 30 *MLL* fusion partners now cloned. Despite this heterogeneity, each translocation

results in the production of a characteristic hybrid protein with an *MLL* amino terminus and a carboxyl terminus, the later encoded by the gene at the partner locus. Fusion transcripts from both reciprocal derivative chromosomes are usually found in leukaemic cells. However clustering of breakpoints found in various 11q23 rearrangements, heterogeneity of breakpoints in the 11q23 fusion partners, and loss of the translocated *MLL* region telomeric to 11q23 have led to the hypothesis that the derived chromosome 11 [der(11)] fusion product, which is encoded by the 5' portion of the *MLL* gene, represents the biologically more relevant potential oncogenic fusion protein (Chen 1991; Cimino 1992; Rowley 1992; Chen 1993). Using highly sensitive reverse transcriptase polymerase chain reaction (RT-PCR) techniques to detect mRNA in peripheral blood and bone marrow *MLL* fusion and duplication transcripts can be detected in the absence of a demonstrable 11q23 rearrangement with or without cytogenetically detectable t(4;11) or other structural chromosomal aberrations involving 11q23 (Chen 1993; Griesinger 1994; Rubnitz 1994; Rambaldi 1996; Rubnitz 1996; Borkhardt 2002).

iii t(12;21)(p13;q22) *TEL/AML1*

One of the more recent additions to the list of chromosomal translocations associated with ALL is the t(12;21)(p13;q22). Abnormalities of the short arm of chromosome 12 (12p) are a frequent finding in haematological malignancies (UKCCG 1992). The translocation was first detected by Romana in 1994 when FISH studies in cases of ALL with abnormalities of 12p detected a cryptic translocation involving the short arm of chromosome 12 and the long arm of chromosome 21, t(12;21)(p13;q22) (Romana 1994). This translocation was subsequently demonstrated to involve the

Ets-related transcription factor *TEL* (*ETV6*) gene at 12p13 and the transcription factor *AML1* (*CBFA2*) gene at 21q22 (Romana 1995). The *TEL* gene, like *MLL*, is frequently rearranged in a variety of hematopoietic malignancies through chromosomal translocation.

This abnormality is the most common translocation in childhood ALL occurring in 25% of B-lineage ALL (Shurtleff 1995; McLean 1996; Borkhardt 1997), with a lower incidence (1-3%) in young adults (Aguiar 1996; Raynaud 1996).

Of interest, is the finding that all positive cases of both children and adults tested by RT-PCR, showed the *ETV6/AML1* transcript, but only a proportion showed the reciprocal one (Shurtleff 1995; Aguiar 1996). This indicated that *ETV6/AML1* was the important product in leukaemogenesis. Driven by the *ETV6* promoter, the *ETV6/AML1* chimeric protein includes the first 336 amino acids of *ETV6* and nearly the entire coding sequence of *AML1*. The DNA-binding Ets domain of *ETV6* is lost in the fusion, suggesting that *ETV6/AML1* does not de-regulate genes normally regulated by *ETV6*.

Deletions of the homologous *ETV6* allele have been reported in the majority of cases with the t(12;21) translocation. This occurred either in all or a proportion of the positive nuclei, and were sometimes intragenic (Raynaud 1996; Romana 1996). The consequence of this biallelic alteration of the *ETV6* gene is complete loss of wild-type *ETV6* function in the leukemic cells. Such a two-hit mechanism is indirectly suggestive of a tumour suppressor function for *ETV6*, although there is no experimental evidence to support this hypothesis. *ETV6*-deficient mice have an embryonic lethal phenotype, making them unsuitable for testing the hypothesis that

ETV6 loss of function alone is leukaemogenic. It has been proposed that the extent of deletion of the second *ETV6* allele and the presence or absence of other chromosomal abnormalities may identify subgroups of *ETV6/AML1* positive patients with different outcomes (Raynaud 1996; Martineau and Greaves 1998).

iv t(1;19)(q23;p13) *PBX1/E2A* (pre-B-cell leukemia transcription factor 1)

The molecular consequence of the t(1;19) is to juxtapose the *E2A* gene at 19p13 with *PBX1* gene at 1q23. The E2A protein is one of the helix-loop-helix family of transcription factors, PBX1 protein utilizes a homodomain for DNA binding. The t(1;19) translocation results in the generation of a chimeric fusion protein consisting of the N-terminal transcriptional activation domain of the E2A fused to the C terminus of PBX1, which contains the DNA-binding homodomain (Mellentin 1989; Nourse 1990).

I:5:2 Gene over-expression

Gene overexpression through chromosomal translocation is a common event in ALL.

i c-*MYC* over-expression

Mature B-cell ALLs usually show one or other of three translocations consistently found in Burkitt's lymphoma t(8;14)(q24;q32) in 80% of cases, t(2;8)(p12;q24) in 6%, or t(8;22)(q24;q11) in 14%. Chromosomes 14, 2, and 22 carry the genes coding for immunoglobulin heavy chain, and light chain kappa and lambda genes respectively. The oncogene c-*MYC* (myelocytomatosis viral oncogene) is normally located at chromosome 8q24. As a result of each translocation, the coding regions

for c-*MYC* gene are brought into close proximity with one of the immunoglobulin genes (Dalla-Favera 1982; Taub 1982; Croce 1983; Erikson 1983; Hollis 1984; Taub 1984). The enhanced expression of c-*MYC* is believed to result from *MYC* coming under the control of these highly active genes and being switched on inappropriately, the over expression of c-*MYC* mediates cellular transformation by disrupting the normal transcriptional programme regulating proliferation, differentiation and cell death in lymphoid cells.

ii *TCRA/D* and *TAL1* over-expression

Most chromosomal translocations associated with T-ALL result in the juxtaposition of transcription factor genes into one of the four T-cell antigen receptor loci resulting in their de-regulated expression. In these translocations the *TCR* gene becomes juxtaposed with other chromosomal sites at the location of putative oncogenes, which then become up regulated. One of the most common translocations is the t(10;14)(q24;q32) bringing the homeobox gene *HOX11* on chromosome 10q24 in close proximity to the *TCRA/D* on chromosome 14q32.

The *TAL-1* gene located on chromosome 1p32 is translocated to the T-cell receptor delta locus at 14q11 in the t(1;14)(q32;q11) translocation seen in 3% of T-ALL leading to the overproduction of the the helix-loop-helix type transcription TAL-1 protein not expressed in normal T-cells (Huang 1995; Stock 1995), more commonly the aberrations of the *TAL1* gene, comprise intrachromosomal submicroscopic deletions resulting in the approximation of the *TAL-1* coding region and the *SIL* promoting region, with increased expression of TAL-1 (Aplan 1992).

1:6 Risk-Adapted Therapies in ALL

1:6:1 Principles of ALL Therapy

The aims of ALL treatment regimens are: the rapid restoration of bone marrow function by using multiple chemotherapy drugs at acceptable toxicities in order to prevent the emergence of resistant subclones, the use of adequate prophylactic treatment of sanctuary sites, such as the central nervous system (CNS); and post-remission consolidation therapies to eliminate minimal (undetectable) residual disease (MRD). Post-remission therapy has traditionally been categorized as intensification or consolidation treatment, and is followed by prolonged maintenance therapy.

For late intensification therapy, many approaches have been used, including bone marrow transplantation (BMT). Although allogeneic BMT may be essential to cure patients with specific high-risk subsets of ALL, or those who relapse after initial treatment (Chao 1991; Barrett 1992; Annino 1994; Forrest 1998) clinical trials do not support its use in first complete remission (CR) for adults with ALL and good-risk or standard-risk features (Horowitz 1991; Zhang 1995). As yet, autologous transplantation has not been proven to be more effective than chemotherapy in ALL (Fiere 1998).

Current treatment strategies in adult ALL result in CR rates of 70- 90%, but long-term disease survival in only 25-50% of patients (GFCH 1996; Secker-Walker 1997; Wetzler 1999).

The ability to identify groups of ALL patients with different prognoses, based on the biology of the malignant clone and clinical patterns of disease, allow treatment to be

tailored to individual risk groups. This approach has resulted in dramatic improvements in outcome for paediatric patients with ALL and as a result risk-adapted strategies based on biological and clinical features are now being applied to adults with ALL in an attempt to improve their survival. Notably, significant progress has been made in survival for adult patients with mature B-cell ALL with the introduction of novel therapeutic approaches for this subset of patients (Hoelzer 1996).

1:6:2 Prognostic Factors

In adult ALL the most important prognostic factors are determined at diagnosis, several clinical and biological factors influence CR rates, remission durations, and survival rates.

In multivariate analyses, patients presenting with WBC counts $>30 \times 10^9/l$ have significantly shorter durations of remission compared with patients with lower leukocyte counts (Kantarjian 1990; Cuttner 1991; Linker 1991; Larson 1995).

Advancing age decreases both CR rate and disease-free survival in almost all adult ALL treatment studies. Age of >60 years is associated with a particularly poor prognosis (Marcus 1986; Kantarjian 1990; Chessells 1998).

Minor factors, or those that have had some significance with certain treatment regimens, are the percentage of circulating blast cells; the degree of bone marrow involvement; the presence of hepatomegaly, splenomegaly, or lymphadenopathy; lactate dehydrogenase levels; and CNS involvement at presentation.

Karyotype is well accepted as an independent prognostic factor in children, and in multivariate analyses of risk factors in adult ALL, karyotype was identified as the most important factor for DFS (Secker-Walker 1997; Faderl 1998; Wetzler 1999).

In general, adult patients with a normal karyotype have improved survival over those with a cytogenetic abnormality. Patients with t(9;22), t(4;11), and t(8;14) [or variant translocations, t(2;8) or t(8;22)] in mature B-cell ALL appear to have better outcomes when treatment approaches other than standard induction, intensification / consolidation and maintenance are used (Secker-Walker 1991; Faderl 1998; Wetzler 1999). Recently, two other cytogenetic abnormalities, -7 and or +8, have been associated with adverse outcomes in adult ALL patients treated with standard multi-agent chemotherapy approaches (Wetzler 1999). In contrast, these studies indicated that patients with a t(10;14) and other abnormalities involving chromosome band 14q11-13, who typically have T-cell ALL, have an excellent prognosis when treated with conventional combination chemotherapy regimens.

In Ph positive ALL to date, allogeneic BMT is the only post remission therapy associated with improved long term DFS for about 30% of these patients (Barrett 1992; Miyamura 1992; Annino 1994).

For patients with an HLA-matched donor, allogeneic BMT in first CR is currently the treatment of choice. Since these patients relapse quickly and are usually resistant to salvage therapy, BMT should be performed as soon as remission is achieved.

In young patients without a donor, as well as the many Ph positive patients who are >60 years old, where allogeneic BMT is not an option, newer strategies including the use of attenuated transplant regimens, with subsequent immunotherapy,

antisense molecules or specific tyrosine kinase that target products of the *BCR/ABL* fusion gene or its chimeric protein product may be considered for this group of high risk patients (de Fabritiis 1998; Ottmann and Hoelzer 2002; Wassmann 2002; Wassmann 2003).

Similarly because of their high risk, pro-B-ALL t(4;11) patients are often offered allogeneic BMT in first CR and this can improve survival up to >60% (Ludwig 1998).

In addition to factors determined at presentation, response to treatment is significantly correlated with outcome. Until recently it was evaluated with relatively coarse parameters such as time to achievement of CR. Failure to attain CR within 4-6 weeks following induction chemotherapy was reported as an adverse prognostic factor (Lazzarino 1982; Hoelzer 1988). In the past decade, new highly sensitive methods for the detection of MRD (disease undetected by microscopic analysis of bone marrow slides), which reached at its best a sensitivity of 1-5 leukaemic cells in 100 normal cells, have been developed. Methods for MRD detection include assessment of leukaemia specific constellation of surface markers by flow cytometry (Orfao 1994; Farahat 1998; Weir 1999), detection of fusion genes related to specific chromosomal translocations, for example detection of the chimeric *BCR-ABL* in t(9;22) by PCR, and analysis of individual rearrangements of immunoglobulin and T-cell receptor genes by PCR (Cole-Sinclair 1993; Stolz 1999; Szczepanski 2002), and more recently by real-time PCR (Eckert 2003). MRD analysis by immunophenotyping may detect one leukaemic cell in 10^4 normal cells, and PCR based studies can detect one abnormal cell in 10^6 normal cells.

Results of MRD evaluation in adult ALL shows that after induction, a high MRD level $>10^3$ was associated with a high risk of relapse (89%), however even patients with low MRD levels $<10^3$ had a considerable risk of relapse (46%) (Brisco 1996; Foroni 1997; Mortuza 2002). In retrospective studies, MRD was a significant predictor of relapse risk at all time points, but its predictive value was improved at later time points (months 6-9). A combination of time points during the first year improved the prognostic model, with 52% of patients with high MRD level ($>10^4$) at any point relapsing compared to none of those with low MRD ($<10^4$) (Brueggemann 2001). These findings have been confirmed in a prospective study (Hoelzer 2001). Using a combination of these recognised risk factors precursor-B or T-ALL allows for stratification of adult patients into three risk groups:

1. Good-Risk Group

This group contains patients with both B and T-lineage disease, who have no adverse cytogenetics, are less than 30 years of age, present with WBC counts of $<30 \times 10^9/l$, and achieve remission in less than four to six weeks. These patients are estimated to have a 3-year DFS of 69% (Linker 1997).

2. Intermediate-Risk Group

This group is comprised primarily of patients with precursor-B ALL <60 years of age. It is likely that as yet undefined, biological features are inherent within subsets of intermediate-risk patients and account for the heterogeneity in DFS rates of this group. The identification of prognostic factors based on disease biology would help more accurately identify the higher risk patients within this group, who would

benefit from dose intensification with allogeneic transplant in first CR. For example, the molecular detection and quantification of MRD following early intensification.

3. High-Risk Group:

This group, characterized by patients with B-lineage disease, presenting with WBC counts $>100 \times 10^9/l$, and/or adverse cytogenetics, has a dismal prognosis with standard treatment approaches, with 3-year DFS estimated between 0 and 20% (Linker 1997). Patients >60 years of age are also considered within this group.

It is thus clear that in adult patients presenting with ALL a comprehensive analysis of risk factors is needed. This must include chromosomal analysis, by conventional cytogenetics where possible or complementary molecular methods.

1:7 Patient selection The MRC Acute Lymphoblastic Leukaemia Trials

The Medical Research Council Leukaemia Steering Committee was formed by interested haematologists, physicians, paediatricians and statisticians in the late 1950's, to consider the treatment of children and adults with acute leukaemia, with a view to establishing randomized trials. The first randomized trial, comparing steroid therapy at high and low dosage in conjunction with 6-mercaptopurine, was published in 1963. The Childhood Leukaemia Working Party initiated the UKALL I trial in August 1970, and reported its first results in November 1972. The Working Party on Leukaemia in Adults set up multicenter trials in acute and chronic myeloid leukaemia, chronic lymphocytic leukaemia, multiple myeloma, and polycythaemia

rubra vera at about the same time, but adult patients with acute lymphoblastic leukaemia were enrolled in the childhood trials, UKALL I through VI. The first entirely adult trials UKALL IX (1985) and UKALL XII (1993) were open to persons over 15 years of age.

UKALL I (1970-1971)

This trial was designed to estimate the effect of prophylactic therapy on the central nervous system (Lilleyman and Eden 1986) and included 16 patients over the age of 15 years in whom the remission rate was 88%. EFS at 5 years, however, was only 6%

UKALL II (1972-1973)

This trial included the addition of L-asparaginase (MRC 1986). Of the 325 patients entered in this trial, 23 were older than 15 years. The remission rate for adults was 70%, EFS at 5 years was 30%, and there were 6 adult survivors at 20 years (26%).

UKALL III (1974-1975)

In this trial two methods of administering L-asparaginase and cytosine arabinoside, and gaps in 6-mercaptopurine administration were assessed in 392 patients, including 37 adults (MRC 1986), in whom the remission rate was 73%, the EFS at 5 years was 14%, and 6 of 37 adults remained alive at 20 years (16%).

UKALL IV (1975-1978), UKALL V (1976-1979)

One hundred and twenty five of 362 patients in UKALL IV were adults who received intensive maintenance chemotherapy (MRC 1986). The remission rate was 79%, the EFS at 5 years was 17% and 23 adults survived at 20 years (18.4%). No adults were entered into UKALL V.

UKALL VI (1978-1980) This trial investigated methods of consolidation therapies. One hundred and fifty one of 379 patients were >15 years old. The remission rate was 87%, the EFS at 5 years was 25%, and 33 patients were still alive at 20 years (22%) (MRC 1985). No adults were entered into UKALL Trial VII.

UKALL VIII (1980-1984)

This trial included randomization at intensification as well as maintenance stages, with over 800 patients recruited over 4 years. It reported the single greatest improvement in EFS seen in children in the UK up to that date (MRC 1986;Eden 1991; Eden 2000). No adults were entered in this trial.

UKALL IX (1980-1985)

This trial ran concurrently with the childhood trial UKALL VIII. A total of 266 patients were entered. The prognostic factors of increased age and high WBC count were identified. The CR rate was 87% and the EFS at 5 years was 26%.

UKALL XA (1985-1992)

This trial was designed to assess the effect on long-term survival of intensifying treatment given during the first four weeks, as reported by the West German Study Group (Hoelzer 1988), to which 618 eligible patients were entered. The remission rate was 88%. At 5 years the overall DFS was 28%, and there was no difference between the three arms of the trial, which contradicted expectations. This was attributed to inadequate intensity of consolidation. Compliance with trial protocol was a problem in this trial, with a number of patients undergoing transplant procedure.

The current trial UKALL XII (1993-to date)

Allografting and autografting were shown to be successful in some patients in UKALL XA. UKALL XII was designed to evaluate the efficacy of BMT in first complete remission and of two highly intensive blocks of therapy after remission. Entry criteria are the diagnosis of ALL in an adult patient (aged 15-55 years at the time of diagnosis), with the exception of the L3 Burkitt type patients, who are treated according to a different protocol.

All patients entered to the trial are expected to undergo full diagnostic workup at presentation, with morphology, cytochemistry, immunophenotyping and cytogenetics as essential requirements. Central review is provided for morphological diagnosis, and the Leukaemia Research Fund (LRF) Cytogenetics Group reviews all regionally analysed karyotypes centrally. (Harrison 2001). The sheer size of participation in this trial (target recruitment at commencement 1000 patients) makes this a perfect setting for collecting information on a number of prognostic factors including full cytogenetic and MRD studies.

1:8 Method selection Screening for chromosomal abnormalities in ALL

1:8:1 Molecular analysis in ALL

Since the prognostic significance of certain chromosomal abnormalities had become known throughout the 1980s cytogenetic analysis became a requirement for entry of patients to trials of treatment. This commenced officially with the UKALLX and XA

trials in children and adults respectively. At that time cytogenetic analysis of haematological malignancies was not well established. In the U.K. there was no standardisation or co-ordination between laboratories and the cytogenetic results in those trials were rather incomplete. In an attempt to improve co-ordination of cytogenetic studies in the following trials, UKALLXI and XII for children and adults, the LRF in association with Professor Lorna Secker-Walker established a cytogenetics group to undertake this role, in collaboration with the United Kingdom Cancer Cytogenetics Group (UKCCG) representatives from 30 laboratories carrying out cytogenetic analysis in acute leukaemia. Through their efforts the cytogenetic results obtained in UKALLXI were much improved (Richards 1998; Hann 2000).

Cytogenetic analysis of leukaemic blasts in ALL is notoriously difficult. There are a number of factors responsible for this: bone marrow aspirates may have low cell counts; in some cases the leukaemic blasts do not divide in culture; the morphology of chromosomes and the quality of chromosome banding may be poor. Failure to yield sufficient analyzable metaphases in 10-30% of cases has been reported in most surveys (Fenaux 1989; Secker-Walker 1990; Schiffer 1992; GFCH 1996; Secker-Walker 1997; Wetzler 1999).

In spite of this, cytogenetic analysis remains the best method for a genome wide detection of all chromosomal abnormalities. Variant or complex rearrangements of established changes can be identified, new recurring abnormalities can be discovered, for which cytogenetics provides the starting point for molecular characterisation of genetic changes. Analysis on an individual cell basis reveals secondary

chromosomal changes involved in karyotype evolution, which may be important in disease progression.

The introduction of molecular analysis throughout the 1980s assisted in overcoming some of the limitations of cytogenetic analysis in ALL. Molecular analysis is highly sensitive for the detection of known genetic abnormalities (Griesinger 1994; Fears 1996; Scurto 1998; Gleissner 2001). It can distinguish lesions that appear identical by karyotyping, but in fact involve different genes with different prognostic and therapeutic implications. It can also detect submicroscopic changes, which may be important in risk assignment (Romana 1994).

The three major molecular techniques applied in the investigation of chromosomally mediated genetic change in ALL are: Southern blotting, reverse transcriptase (RT)-PCR and FISH. Southern blotting and RT-PCR have the advantage that they can be carried out on DNA or RNA prepared from the total cell population of leukemia samples and do not require the preparation of cells in metaphase. Translocations that result in breakage within a well-defined region of a gene can be investigated by Southern blotting, for example in *MLL* gene rearrangements, in which there are numerous partner genes many of which have not as yet been cloned. RT-PCR is widely applicable for the rapid and sensitive detection of translocations, provided both partner genes have been cloned and the breakpoint clusters are small enough to design specific primers. It is an excellent method for the detection of MRD (Gehly 1991; Lion 1992; Miyamura 1993; Satake 1997; Park 2000). RT-PCR is limited by the need for viable cells for RNA extraction and the need for multiple primers in multiplex experiments, to allow the reliable detection of translocations with more

than one breakpoint such as the *BCR/ABL* translocations and *MLL* gene rearrangements. Insufficient primers give false negative result. On the other hand, the exquisite sensitivity of the technique makes contamination with a few cells, or even a trace of DNA a potential source of false positive results (Bagg and Kallakury 1999). FISH in interphase and metaphase has a number of advantages over molecular analysis in the detection of prognostically important chromosomal abnormalities. The availability of a wide range of robust probes and refined technologies for application and image analysis particularly, when applied in a complimentary manner with conventional cytogenetic techniques, means that FISH is the best method for the detection of both numerical and structural chromosomal abnormalities in ALL, the rapid detection of which allows the modification of treatment modalities offered to patients.

The childhood trial, ALL 97 was established in April 1997. It had a high-risk arm, in which patients were treated according to a different protocol. These included patients with the translocation, t(9;22), *MLL* gene rearrangements, and near haploidy. In order to ascertain that these patients were assigned to the correct risk group, in addition to cytogenetic analysis that was being carried out on >95% of patients, material from cytogenetic preparations was additionally used in an interphase FISH screening programme to detect these chromosomal abnormalities. This also allowed an assessment of the effectiveness of this approach to be compared with that of conventional cytogenetics for accuracy of detection. Testing for *BCR/ABL*, *MLL* rearrangements and the cytogenetically invisible *ETV6/AML1* were carried out in all cases. Near haploidy was tested for using centromeric probes

in cases with normal or failed cytogenetic result (Harrison and Foroni 2002).

1:8:2 Fluorescence *in-situ* hybridization:

FISH relies on the ability of fluorescent molecules to be deposited in chromatin at the sites of specific DNA sequences. The use of the *in situ* hybridization technique began in the late 60s, when Gall and Pradue reported the hybridization of radioactive DNA probes for repetitive sequences to mouse and drosophila chromosomes. Harper who mapped the insulin gene to chromosome 11p15, achieved autoradiographic localization of single genes on human chromosomes (Harper 1981). Methods for non-isotopic *in situ* hybridization and detection were soon developed, to overcome the difficulties of the use of radioactive probes. The technique was refined throughout the 1980's until by the mid 1990's 24 different chromosome painting probes specific for each of the somatic chromosome pairs and the sex chromosomes, X and Y, could be hybridized simultaneously and detected in different colours using specialized filter sets and computer software (Speicher 1996; Harrison 2000; Van Limbergen 2002).

The components of the FISH procedure are the probe (DNA or RNA) of interest, which can range in size from a single gene to whole chromosomes, and the locus i.e. the target sequence, to which the probe hybridizes. In order to microscopically visualize the hybridization of the probe to the target, a fluorescent tag is attached to the probe. The probes for the identification of individual chromosomes, or chromosome arms, are often called painting probes or whole chromosome paints (wcps). For the detection of deletions or duplications, probes are derived from the gene sequence in question or from closely linked markers, the so-called single copy

gene probes. Also useful are repetitive sequences probes of structural regions of chromosomes for example those to alpha satellite regions near centromeres can be used to identify and count individual chromosomes. Subtelomeric probes may be used to define cryptic translocations. The probe is labelled, either directly by the incorporation of a fluorochrome-conjugated nucleotide e.g. fluorescein, or indirectly with the incorporation of a reporter molecule e.g. biotin-dUTP or digoxigenin-dUTP.

Principles of the FISH method:

Target sequences are prepared, the probe and target sequences are denatured with heat and/or formamide, they are then combined and allowed to hybridize.

Non-specifically bound probe is washed off the target. If the probe was directly labelled with a fluorochrome, antifade solution with a counter stain such as propidium iodide (PI) or 4'6-diamino-2-phenylindole dihydrochloride (DAPI) is applied and the preparation is cover slipped ready for analysis.

Indirectly labelled probes are detected by a reaction that binds a fluorochrome e.g. fluorescein-avidin or antidigoxigenin-rhodamine to the probe.

If the target sequence is large, >50kb, the signal is usually visible. Smaller signals may need to be amplified by immunocytochemistry. (Fig I:1)

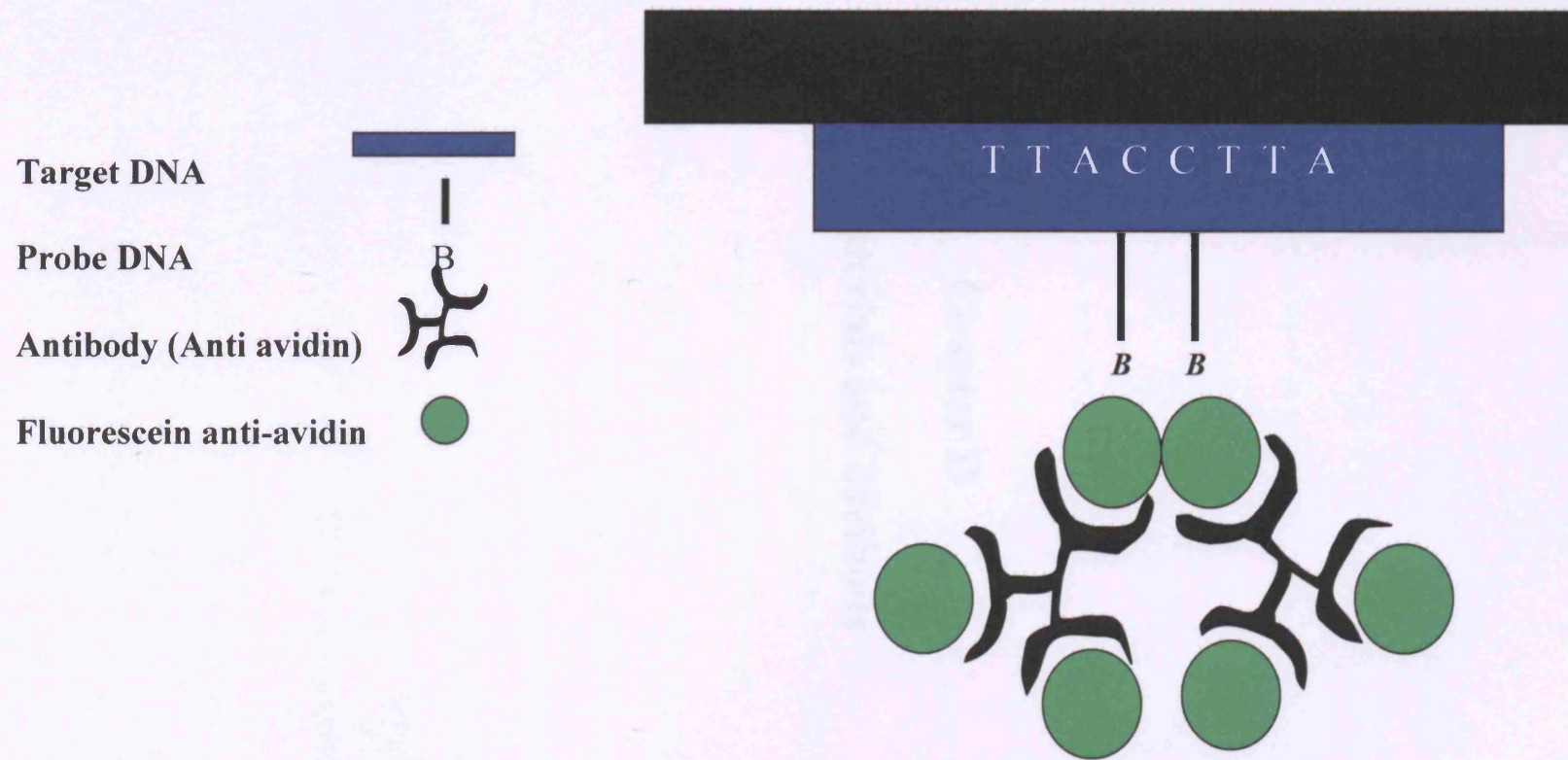


Fig I:1. Principle of FISH

Chapter II

Materials and Methods

II: 1 Patients and controls

Patient selection:

The work presented here commenced in October 1998. Regional cytogenetics laboratories carrying out cytogenetic studies on patients entered to the UKALLXII trial were informed in writing of the commencement of the study and asked to send fixed cell suspensions from cytogenetic preparations from newly diagnosed adult patients with ALL. This included trial and non-trial patients.

Respondents sent new and retained samples from 185 patients, or information on studies carried out locally in a further 8 patients with adult ALL. Samples were blood in 14 cases, bone marrow in 172 cases, and both in 7 cases, of which 178 samples were from patients entered to UKALLXII.

Patient samples were assigned unique identification numbers (UIN) (from 1-193).

These UIN are presented in all tables and images within this work.

Clinical features of all patients are shown in Table II: 1. With the exception of one patient, the age range was that of inclusion to the UKALLXII trial i.e. 15-55 years, the sex ratio of 1.35:1 male: female was similar to that reported in other adult series. Considering the largely retrospective nature of the study and dependence on availability of stored material, the proportion of cases with failed cytogenetics (fewer than 20 normal metaphases analysed), at 22% was only slightly higher than expected for a multicentre trial.

Nine bone marrow samples were selected as controls from adult patients with a normal karyotype and non-malignant haematological disease. These included six

cases with immune thrombocytopenia, two with iron deficiency anaemia and one with immune neutropenia.

For chromosomal enumeration experiments using the Multiprobe®-I (as detailed in section II:4) five bone marrow samples were selected as negative controls, two from patients with a normal karyotype and non-malignant haematological disease; one with immune thrombocytopenia, and the other with immune neutropenia, and three cases from adults with ALL with pseudiploidy, as well as two positive controls from patients with ALL and hyperdiploidy.

Table II.1 Clinical features of patients in the study.

Age	Sex	WBC x10 ⁹ /l	Immunophenotype					Conventional		
			c-ALL	Pre-B	T-cell	Null	Other	Cytogenetics		
Mean	M: F	Median						Abn	Norm	Fail
(Range)		(range)								
31	111:82	9	82	34	32	3	11	111	39	43
(15-88)		(0.11-930)								

cALL: CD10+Common ALL analysed Abn :Abnormal clone identified Norm:20 normal metaphases

II: 2 Cytogenetics

Cytogenetic analysis was carried out on diagnostic samples in the UK regional cytogenetic centres. All samples were processed by established short-term culture methods (Secker-Walker 1997). The G-banded slides and karyotypes were reviewed by the LRF Cytogenetics group and collected into their database. Karyotypes were described according to the International System of Human Cytogenetic

Nomenclature (Mitelman 2004). The requirement for a classification of “normal karyotype” in the UKALLXII trial is complete analysis of 20 metaphases.

II: 3 FISH slide preparation:

Fixed cell suspensions used for conventional cytogenetics were stored at -20°C , and re-used for the FISH studies.

Successful FISH is dependent on the quality of the fixed cell suspension available. Isolated nuclei without residual cytoplasm or other cellular debris are vitally important, since the presence of these debris reduces the intensity of FISH signals and increases background staining in both interphase and metaphase studies, but more markedly in metaphase FISH studies.

Numerous methods have been described to optimise the quality of FISH preparations, utilizing a combination of factors such as temperature gradients, positional factors (such as distance between dropper and slide, and placing the slide at an angle) and humidity as well as various enzymatic techniques to remove cellular debris and cytoplasmic residues. In this study I adopted the following steps for slide preparation for FISH.

1-Stored fixed bone marrow/peripheral blood samples were stored at -20°C in 3:1 Methanol: Acetic acid fixative.

2-The cells were washed and resuspended in fresh fixative solution prior to slide preparation.

3-The cell suspension was dropped from a height of about 15cm onto an ice-cold slide placed on a rack over boiling water to create the necessary humidity, and

facilitate spreading and flattening of cells.

4-As the fixative started to evaporate from the edges of the drop; a further drop of ice-cold fixative solution was added.

5-The slide was placed in an air current to speed up the evaporation of the fixative.

6-Further flattening and separation of the interphase cells and chromosomes within the metaphases was achieved by placing the slide on a hot plate at 60⁰C for a few minutes to dry completely.

7-Any residual cellular debris or cytoplasm was removed by putting the slides into a 70% solution of glacial acetic acid, at room temperature for 1-2 minutes, followed by rinsing in distilled water for a further 2 minutes.

8-The slides were then aged in 2 x SSC (sodium chloride, sodium citrate) for 30 min at 37⁰C, dehydrated in an ethanol gradient at 70%, 90% and 100% for 2 minutes each and air-dried.

II: 4 FISH Probes & Equipment

II: 4:1 Probes

Commercially available directly or indirectly labelled probes were used throughout the study. Single or dual colour probes were used for the detection of *BCR* and *ABL*, *MLL*, *ETV6* and *AML1* rearrangements. (Vysis, U.K. Oncor, France) Centromeric probes (Oncor, France), and a device coated with 24 individual directly labelled alpha satellite probes (Chromoprobe Multiprobe®-I, Cytocell UK) were used for chromosome enumeration and marker chromosome identification. Confirmation of translocations and marker chromosomes was carried out using directly labelled

whole chromosome painting probes (Cambio, STARTM FISH, U.K.)

(i) BCR/ABL probes:

The probes used to detect the BCR/ABL fusion of the t(9;22)(q34;q11) translocation:

A LSI Dual Colour BCR/ABL probe (Vysis, U.K.)

This dual colour probe contains two probes; the *BCR* probe extends from between *BCR* exon 13 and 14 (M-bcr exons 2 and 3) in a centromeric direction on chromosome 22, approximately 300kb, extending beyond the m-bcr region. The *BCR* probe spans both M-bcr and m-bcr breakpoints, thus detecting breakpoints in both regions. The probe for *ABL* begins between exons 4 and 5 and extends over 200kb towards the telomere of chromosome 9, spanning the breakpoint region. The probes are directly labelled with SpectrumGreen (BCR) and SpectrumOrange (ABL).

In a normal cell two red and two green signals are observed. As a result of the translocation the *ABL* probe becomes juxtaposed to *BCR* on the der(22) to provide a yellow fusion signal in metaphase and interphase cells, in association with one red and one green signal.(Fig II:1) In a nucleus possessing the t(9;22) involving the M-bcr, one green (native *BCR*), one red (native *ABL*), one red/green (yellow) (fused 5'BCR/3'ABL) signal are observed. Minor breakpoint (m-bcr) signal patterns appear as one red, two green and one fusion signal.

B LSI Dual Colour BCR/ABL ES (Extra signal) probe (Vysis, U.K.)

This probe detects *BCR/ABL* fusion with increased sensitivity, it contains a *BCR* probe similar to that in the *BCR/ABL* probe kit, and an *ABL* probe that extends

beyond the breakpoint cluster region to include the Argininosuccinate synthetase (*ASS*) gene sequences (ES probe). In *BCR/ABL* positive cells, in addition to the fusion signal, an extra red signal from the residual *ASS* area of the derived chromosome 9 will be seen, cells without this second signal are negative, thus reducing the false positive rate. (Fig II:2)

(ii) LSI Dual colour *TEL/AML1* LS probe (Vysis):

This probe is used to detect the *ETV6/AML1* fusion of t(12;21)(p12;q22) translocation. The probe for *ETV6* extends from between exon 3-5 towards the telomeric end of chromosome 12, covering approximately 350 kb. The probe for *AML1* extends over 500 kb spanning the entire gene. The probes are directly labelled with SpectrumGreen (*ETV6*) and SpectrumOrange (*AML1*). Normal cells have two red and two green signals, in the interphase nuclei of *ETV6/AML1* positive cells, one or more fusion signals are seen representing the fusion gene on the derived chromosome 21. Since the *AML1* probe spans the breakpoint, the signal splits between the derived chromosomes 12 and 21 so that in addition to the fusion signal with the *ETV6* on the derived chromosome 21, a small extra signal is present on the derived chromosome 12. A red signal corresponding to the normal *AML1* signal on chromosome 21 and a green signal corresponding to the normal *ETV6* on chromosome 12 are seen. (Fig II:3).

(iii) *MLL* probes

These probes were used to detect translocations involving the *MLL* gene at 11q23

A Single colour *MLL* probe (Oncor)

This is an indirectly labelled probe, which spans the *MLL* breakpoint cluster region. In cases with a rearrangement of *MLL* the probe is split between the derived chromosomes, giving three signals in positive interphase cells. (Fig II:4) Cases with three or more signals need further testing using centromeric probes to exclude trisomy for chromosome 11. Patients with deletions, duplications, loss or gains of one signal will be detected. This probe does not detect breakpoints 3' of the breakpoint cluster region which occur in 20% of cases.

B LSI Dual colour *MLL* probe (Vysis, U.K.)

This comprises two directly labelled probes, located to 11q23, The SpectrumGreen labelled probe lies centomeric to *MLL* and SpectrumOrange probe telomeric to the *MLL* breakpoint. In normal cells there will be two yellow (fused or closely adjacent green and red) signals. Cells with an *MLL* translocation will show one yellow signal (on the normal 11q23) and separated red and green signals (one on each of the derived chromosomes). (Fig II:5) The probe successfully detects unbalanced translocations in which one (usually the red) of the separated signals is lost.

(iv) Centromeric probes:

FISH testing for numerical chromosomal changes was carried out using the Multiprobe®-I device. This device allows detection of 24 centromers in a single hybridisation on one slide. (Fig II:6) The probes used in the Multiprobe®-I kit are listed in (Table II:2).

Alternatively the gains or a loss of individual chromosome was assessed using indirectly labelled alpha satellite probes (Oncor) in single or dual colour combinations.

(v) Whole chromosome painting:

Whole chromosome painting probes (wcps) (Star*FISH, Cambio) directly labelled with biotin or Cy3 were used.

Table II.2 Probes used in Chromoprobe Multiprobe®-I

Multiprobe template square	Chromosome	Locus	DNA class
1	1qh	D1Z1	satellite III
2	2	D2Z2	α -satellite
3	3	D3Z1	α -satellite
4	4	D4Z1	α -satellite
5	1/5/19	D1Z7/D5Z2/D19Z3	α -satellite
6	6	D6Z1	α -satellite
7	7	D7Z1	α -satellite
8	8	D8Z2	α -satellite
9	9qh	D9Z3	satellite III
10	10	D10Z1	α -satellite
11	11	D11Z1	α -satellite
12	12	D12Z3	α -satellite
13	13/21	D13Z1/D21Z1	α -satellite
14	14/22	D14Z1/D22Z1	α -satellite
15	15	D15Z4	α -satellite
16	16	D16Z2	α -satellite
17	17	D17Z1	α -satellite
18	18	D18Z1	α -satellite
19	1/5/19	D1Z7/D5Z2/D19Z3	α -satellite
20	20	D20Z1	α -satellite
21	13/21	D13Z1/D21Z1	α -satellite
22	14/22	D14Z1/D22Z1	α -satellite
X	X	DXZ1	α -satellite
Y	Y	DYZ1	satellite III

II: 4:2 Equipment

Slides were analysed using a Ziess Axiophot epifluorescence microscope (Ziess, Oberkochem, Germany) equipped with a 100W mercury lamp. Images were

captured using a cooled coupled device (CCD) camera (Photometrics) and analysed using Mac Probe software (Applied Imaging International Ltd, UK).

II: 5 FISH techniques

II: 5:1 Directly labelled commercial probes

(LSI probes; *BCR/ABL*, *MLL*, *ETV6/AML1* and directly labelled whole chromosome paints)

Method A

1-Denaturation of target DNA was achieved by flooding the selected area of the slide with 40µl of 65% Formamide, slides were heated on a hot plate to 72⁰C for 2min, then dehydrated in an ethanol gradient at 70%, 90% and 100% and air dried.

2-Locus specific probes were reconstituted in formamide, in an eppendorf to which 1µl of probe, 2 µl distilled water and 7 µl of Hybrisol VII (100% formamide) were added. The solution was heated in a water bath to 72⁰C for 5 minutes, to denature the probes.

3-WCP probes were reconstituted by adding 13 µl of Hybrisol VI (65% formamide) to each 2 µl of probe, denatured by heating in a water bath to 72⁰C for 10 minutes, re-annealed by incubation at 37⁰C for a further 30 minutes.

4-The probe was applied to the slide, the hybridization area was covered by a glass coverslip, the edges of which were sealed with cow-gum. The slides were hybridized overnight in a humid chamber at 37⁰C. (A plastic slide box kept in an incubator, moisture was created by placing wet paper towels under the slides).

5-Non-specifically bound probe was removed the next day by washing the slides in a 0.5XSSC solution, heated to 72⁰C for 2 minutes, followed by a second wash in a mixture of 2XSSC + Tween, at room temperature for a further 2 minutes.

6-Slides were counter stained with DAPI, and mounted in antifade solution (Vectashield; Vector laboratories, Burlingame, CA, USA).

Method B co-denaturation

In this modified approach target DNA and probe were denatured together.

Reconstituted probe was applied to the target area, slides and probe were co-denatured on a hot plate at 72⁰C for 2 minutes. Cohybridisation and washing steps was carried out as described in steps 4-6 above.

II: 5:2 Indirectly labelled probes

(Single colour *MLL* specific probe and alpha satellite centromeric probes)

1-Denaturation of the target DNA was achieved by flooding the area of the slide with 40µl of 65% Formamide. Slides were heated on a hot plate to 72⁰C for 2 min, then dehydrated through an ethanol gradient at 70%, 90% and 100% and air-dried.

2-The probe was reconstituted by heating to room temperature (*MLL*) or reconstituted with formamide by adding 1µl of probe to 15µl of Hybridsol VI (65% formamide) and denatured by heating in a water bath to 72⁰ for 5 minutes (alpha satellite centromeric probes).

3-The probe was applied to the target area of the slide, covered with a glass coverslip and sealed with cow-gum. The slides were hybridized overnight in a humid chamber at 37⁰C.

4-Non-specifically bound probe was removed the following day by washing slides in a 0.5XSSC solution heating to 72⁰C for 2 minutes, followed by a second wash in a mixture of 2XSSC + Tween, at room temperature for a further 2 minutes.

5-Detection of probe was achieved by flooding the target area with 30μl of antidigoxigenin-rhodamine/ antiavidin- Fluorescein isothiocyante (FITC) mixture and incubating the slide for 15 minutes at 37⁰C.

6-Excess fluorochrome bound antibody was removed by washing slides in a mixture of 2XSSC + Tween, at room temperature for 2 minutes before counterstaining and analysis as described above.

II: 5:3 Multiprobe®-I

1-1-2μl of fixed cells were dropped onto each of the 24 scored squares on the customizes slide (Fig II:6) cell density was adjusted to ensure at least 100 nuclei in each square.

2-1μl of hybridization solution (65% formamide) was added to the probe attached to each of the squares of the coverslip device.

3-Alpha satellite probe and target DNA were then co-denatured by placing the slide over the coverslip; the whole device (slide and coverslip) was then inverted and placed onto a hot plate at 75⁰C for 2 minutes.

4-The device was hybridized in a humid chamber (a plastic slide box suspended in a water bath) at a temperature of 37⁰C overnight.

5-The next day the slide was washed in a solution of 0.4XSSC heated to 70°C for 2 minutes, followed by a further wash in a mixture of 2XSSC and Tween at room temperature for a further 2 minutes.

6-Slides were then counter stained with propidine iodine and mounted in antifade solution (Vectashield; Vector laboratories, Burlingame, CA, USA).

II: 6 Triple testing or Multiplex FISH

The method was adapted to combine the three dual colour probe tests for *BCR/ABL*, *MLL*, and *ETV6/AML1* rearrangements in a single hybridisation step on one slide, in a triple or multiplex FISH test. In the presence of good quality cellular sample a slide was spotted with three separate drops of suspension, each equivalent to 3µl as shown in (Fig II:7). Probes were reconstituted as described above, with one third of the probe volume added to each test area. The three areas were hybridised under separate 13 mm diameter coverslips overnight. Using this modification it was possible to greatly reduce the time, volume of sample used and the quantity of probes utilised.

II: 7 Sequential FISH

Material for this group of adult ALL patients was limited and often of poor quality, therefore to conserve these valuable cells, we developed a method of serial hybridisation of probes (sequential FISH). Wcp were applied to slides following their hybridisation with locus specific probes.

After examination and image capture of all required cells,

1-The coverslips were removed by immersion in phosphate buffer solution (PBS) at room temperature for 15 minutes.

2-The slides were then dehydrated through an ethanol gradient.

3- wcp directly labelled with biotin or Cy3 (Oncor) were used in dual (using one biotin and one Cy3 labelled probe) or triple colour (one biotin, one Cy3 and the third composed of equal volumes of biotin and Cy3 giving a yellow fluorescence) combinations.

Up to five serial hybridisations with wcp for two or three chromosomes at a time were carried out. Following each hybridisation slides were washed and dehydrated as described above.

4-Images were captured following each sequential hybridisation. The serial digital images were superimposed using MacProbe software; each chromosome was assigned a different pseudocolour. Thus a complete image of the metaphase with variously coloured chromosomes was created in each case, to enable improved interpretation of chromosome abnormalities.

Where metaphases were very limited, in samples with unidentified chromosomes, a further modification to this technique, by the application of wcp to interphase nuclei.

The presence of more than two distinct areas of hybridisation within nuclei suggested that translocations involving large segments of the painted chromosome were present. Once the number of potential chromosomes involved in translocations had been determined, their origins could be confirmed by sequential metaphase FISH.

II: 8 Determination of positive results and cut-off values

For locus specific dual-colour FISH probes, 200 nuclei were scored for each test wherever possible. A minimum of 100 cells was accepted for a test result to be eligible for inclusion in this study. The number and the co-localization of the red and green signals were determined for each nucleus. A positive result from co-localization of the red and green fluorescence was accepted only if a yellow fusion signal (produced by overlapping red and green signals) was observed. Touching red and green signals were considered to be negative. The proportion of cells with co-localized signals was calculated as a percentage of the total number of nuclei analysed testing with each probe and for each patient.

To determine the hybridisation efficacy for each probe the nine control samples were hybridised individually with each probe as described above. Four hybridisation signals, two specific for *BCR* and two for *ABL* were found in $96 \pm 1.3\%$ of interphases examined, two specific for *ETV6* and two for *AML1* were found in $96.5 \pm 2\%$ of interphases examined. Two hybridisation signals were found in $96.7\% \pm 1.7$ of nuclei tested with the *MLL* Oncor probe, and in $97.1\% \pm 2.3$ of nuclei tested with the *MLL* Vysis probe. The results indicate high hybridisation efficiency for all three-probes.

In normal nuclei two discrete signals for each *BCR* and *ABL*, *ETV6* and *AML1* should be seen. As a result of close positioning of two signals by chance it is possible to detect apparent fusion signals (false positive results) in a small proportion of normal nuclei, a mean rate of fusion signals of $1.76 \pm 0.97\%$ was

found in the nuclei of the normal controls tested for *ETV6/AML1* fusion, and $1.56 \pm 0.86\%$ in nuclei tested for *BCR/ABL*. Thus the cut-off point for false-positive nuclei was set at 4.67% for *ETV6/AML1* and 4.1% for *BCR/ABL* (calculated from the mean of the nine control samples plus 3x standard deviation (s.d)).

The single colour *MLL* probe showed three signals in $1.3 \pm 1.4\%$ of nuclei and the cut-off point for false positive nuclei was set at 5.5% (mean, plus 3x s.d.). The dual colour *MLL* probe showed a separation of fused dual colour signals in split signal in $0.69 \pm 0.7\%$ of nuclei and the cut-off point for false positive nuclei was set at 2.79% (mean, plus 3x s.d.).

For the Chromoprobe Multiprobe®-I, one hundred nuclei were counted for each probe. The five control samples were hybridised as described above, hybridisation efficiency of individual centromeric probes was hugely variable, the proportion of nuclei with strong hybridisation signals ranging from 47-94% as detailed in (Table II: 3).

The proportion of nuclei showing disomy was also variable, ranging from 83-98% as shown in (Table II: 4). The cut-off points for monosomy (range from 3-30%) and trisomy (range from 1.5-29%) calculated from mean +3x s.d. using nuclei with abnormal numbers of signal are also shown in this table.

The centromeric probe for chromosome 5 cross-hybridises to chromosome 1 and 19 making reliable analysis difficult. Less than 50% of normal control samples showed the expected six signals, thus further analysis was not carried out using this probe.

In every case a number of probes failed to provide a satisfactory result, either due to insufficient analysable nuclei, poor hybridisation or excessive cross hybridisation.

Only cases with a minimum of 10 analysable squares were included in this study.

Probes which failed most frequently were chromosomes 1, 20, 4, 14/22, X, and 13/21, the proportion of cases where each probe failed are shown in (Table II: 5).

Table II.3 Hybridization efficiency of probes in Chromoprobe Multiprobe®-I

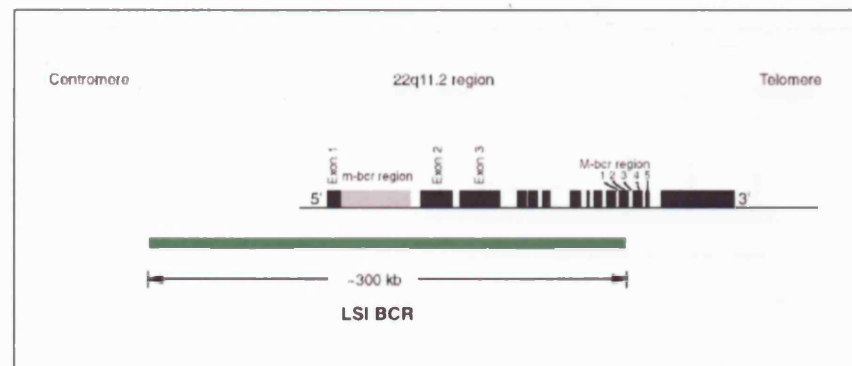
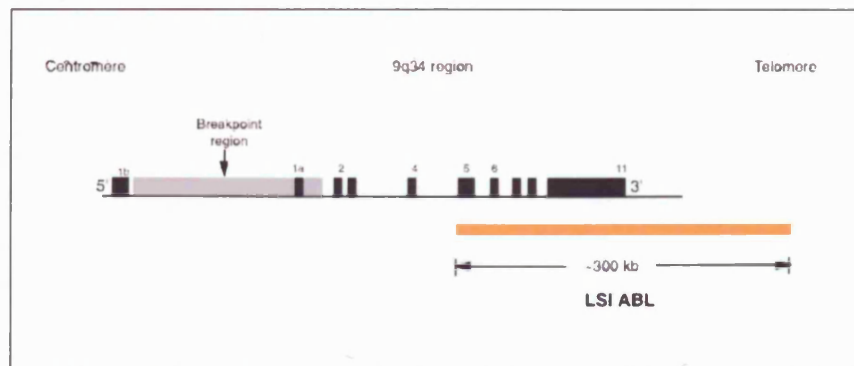
Probe for Chromosome	Nuclei with hybridisation (% of nuclei examined)
1	47%
2	88%
3	59%
4	82%
1/5/19	NA
6	83%
7	79%
8	91%
9	90%
10	88%
11	53%
12	85%
13/21	79%
14/22	71%
15	82%
16	88%
17	63%
18	74%
20	58%
X	94%
Y	77%

Table II.4 Chromoprobe Multiprobe®-I findings in control samples

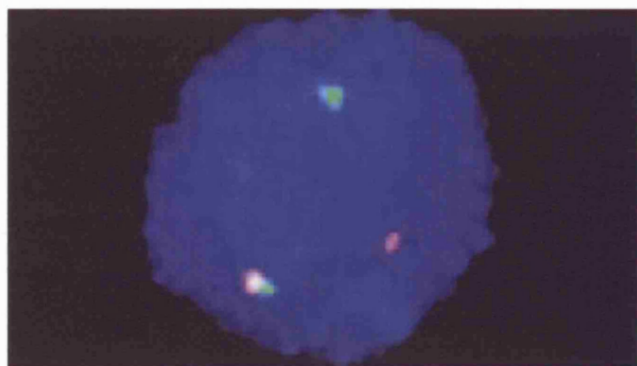
Probe for Chromosome	Frequency of signals per nucleus % and cut-off point for normal reading (mean+3x s.d.)											
	1 signal	Cut-off point	2 signals	Cut-off point	3 signals	Cut-off point	4 signals	Cut-off point	5 signals	Cut-off point	6 signals	Cut-off Point
1	3.73±3.16	13.2	95.2±3.3		1.06±0.11	1.4						
2	1.3±0.6	3.1	98±1		0.63±0.55	2.3						
3	2.33±2.52	9.8	95±1.73		2±1	5	0.66±1.15	4.1				
4	4.16±4.25	16.9	94.6±4.9		1.3±0.64	3.2						
6	2.66±1.52	7.3	95.7±3.5		1.33±1.53	5.9						
7	3.23±0.68	5.3	94.3±0.57		1.63±1.18	5.2						
8	1.23±1.36	5.3	98.1±1.65		0.66±1.15	4.1						
9	1±2	7	98.5±1.71		0.5±2.59	8.2						
10	1.9±2.47	9.3	96.9±2.32		0.59±0.54	2.2						
11	5.45±2.9	14.1	92.7±2.8		2.43±0.98	5.4						
12	2.28±0.53	3.9	97.1±0.78		1±0.1	2.5						
13/21			0.75±1.5	5.2	15±6.37	34.1	83.25±5.5		3.66±3.51	14.1	0.25±0.5	1.75
14/22			0.5±1	3.5	17.3±3.86	28.9	83.5±3.32		1±1	4		
15	8.95±4.15	21.4	90.8±3.8		0.7±1.1	3.9						
16	3.78±3.82	15.2	95.44±3.7		0.56±0.87	3.2	0.2±0.44	1.5				
17	4.97±1.18	8.5	95±2.65		0.6±0.89	3.3	0.2±0.44	1.5				
18	3.25±2.06	9.4	97.8±4.15		0.6±0.55	2.24						
20	8.3±7.35	30.6	90.05±8.8		1.8±1.27	5.6						
X(Female)	1.21±1.35	5.2	98.1±1.74		0.71±1.5	5.2						
X(Male)	95.6±1.53		3.5±2.1	9.7								
Y	97.25±2.5		3.7±2.1	9.9								

Table II.5 Failure rates of individual centromere probes in the Multiprobe-I

Probe for Chromosome	1	2	3	4	6	7	8	9	10	11	12	13/21	14/22	15	16	17	18	20	X	Y
Failed cases (% of total)	60	0	12.5	48	12.5	10.4	4.2	6.25	10.4	16.7	12.5	33.3	44	16.7	10.4	12.5	12.5	48	37.5	11.4



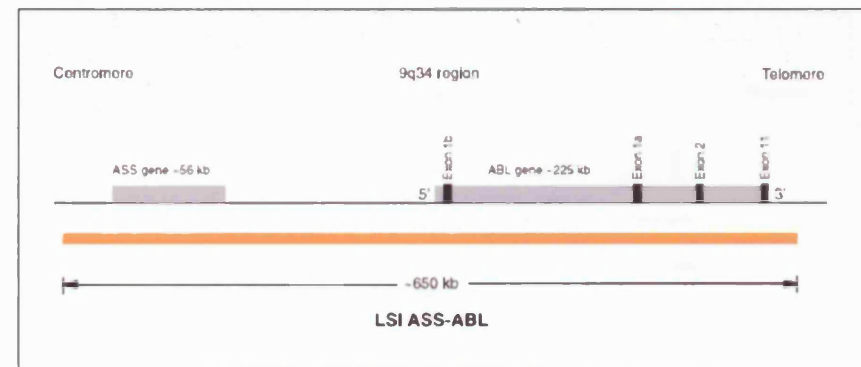
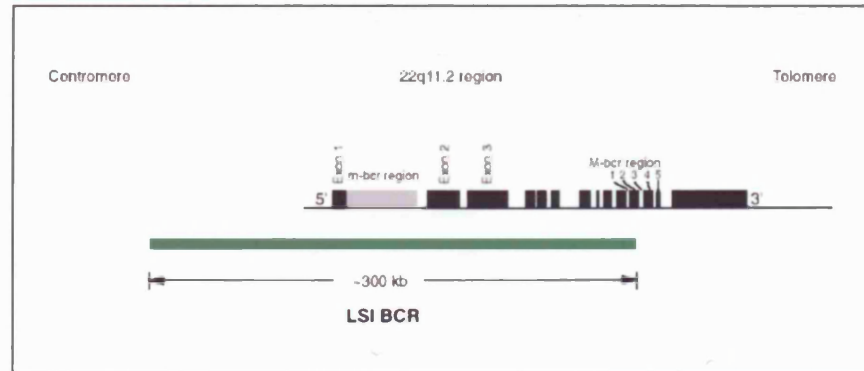
Probe map



Probe image

LSI BCR/ABL Dual colour, single fusion probe hybridised to a nucleus with the t(9;22). Showing one red, one green and one yellow fusion signal pattern (1RIGIF)

Fig II:1 Dual colour BCR/ABL LS probe



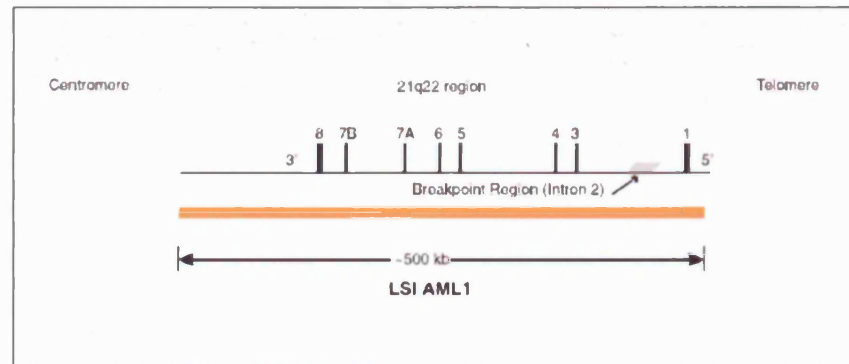
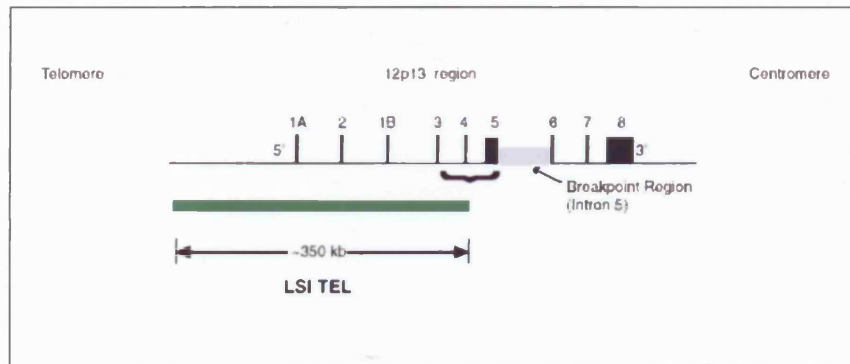
Probe map



Probe image

LSI BCR/ABL ES Dual colour probe hybridised to a nucleus with the t(9;22) Showing one green (native BCR), one large red (native ABL), one smaller red (ES) and one yellow fusion (2RIGIF) signal pattern.

Fig II:2 Dual colour BCR/ABL LS ES probe



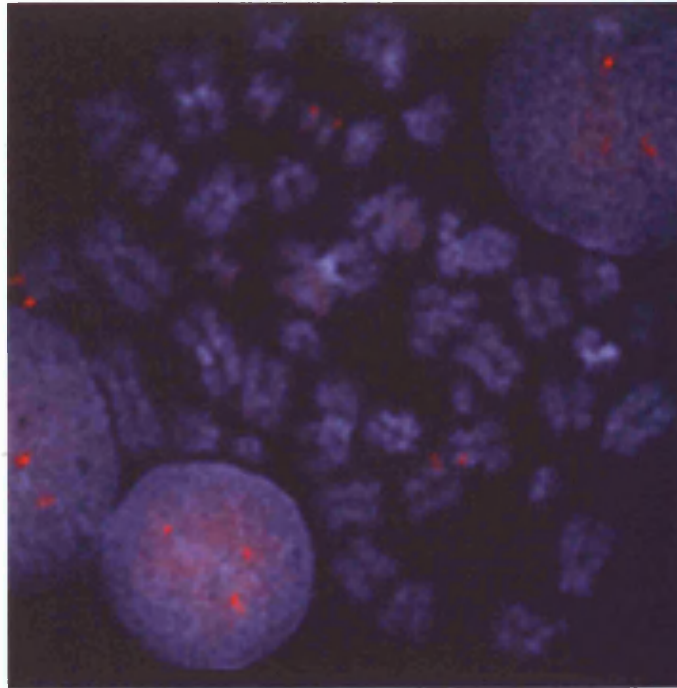
Probe map



Probe image

LSI ETV6(TEL)/AML1 Dual colour probe hybridised to a nucleus without the t(12;21) translocation showing two red and two green signal pattern (2R2G).

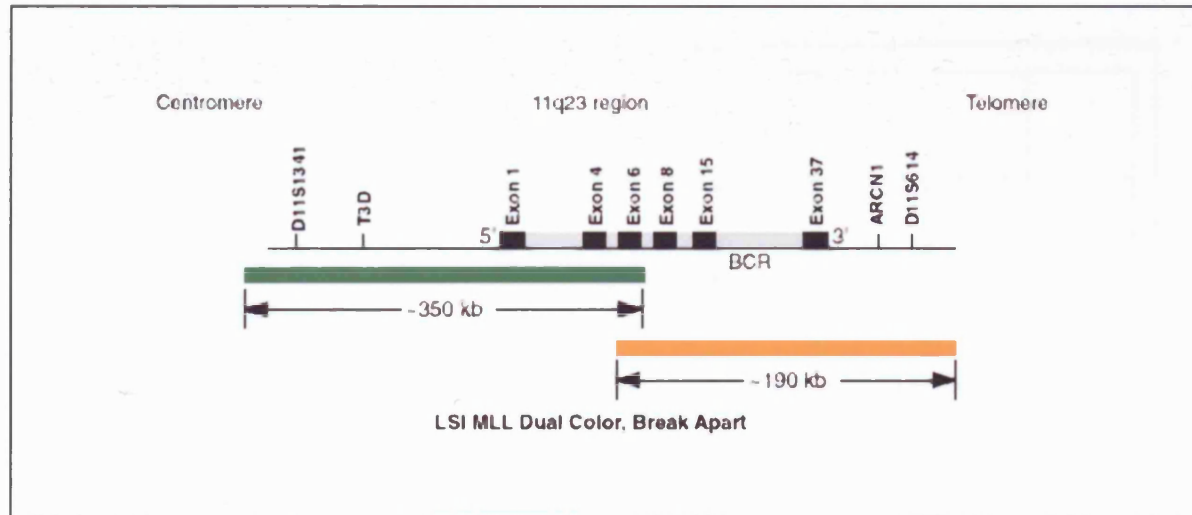
Fig II:3 Dual colour ETV6/AML1 LS probe



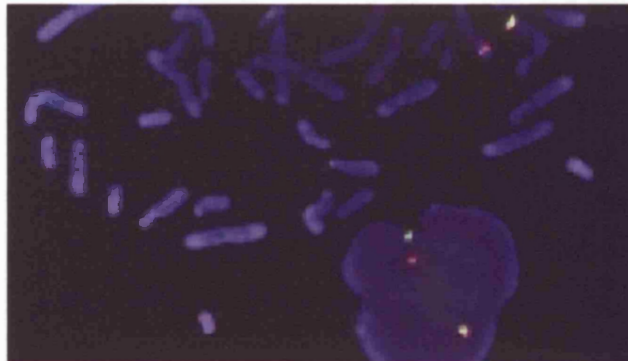
Probe image

LSI MLL single colour probe hybridised to cells with a t(9:11)(p22;q23) showing three red signal pattern.

Fig II:4 Single colour MLL probe



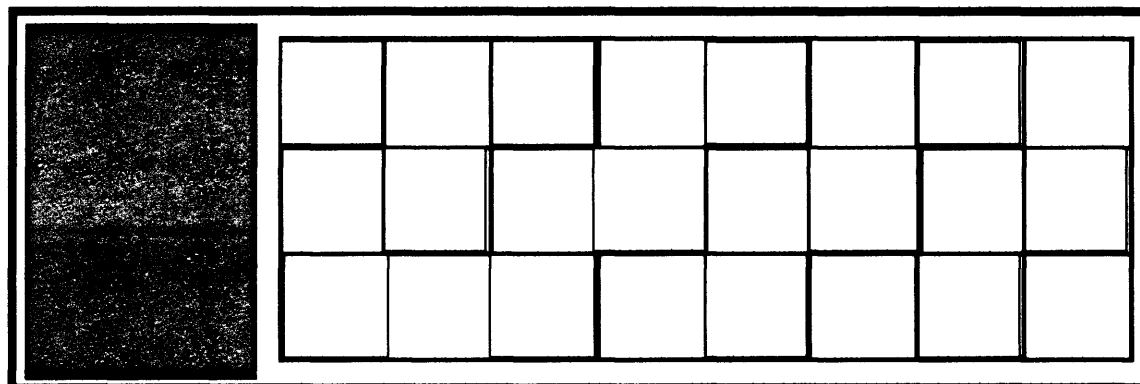
Probe map



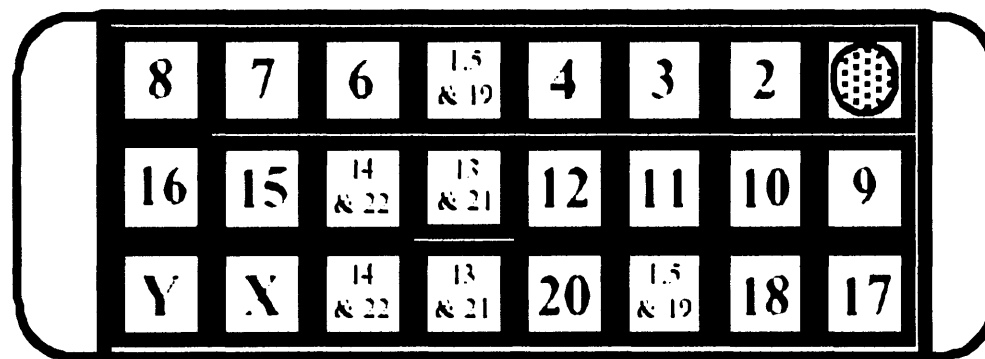
LSI MLL Dual colour probe hybridised to cells with a t(9;11)(p22;q23) showing one red, one green and one yellow fusion signal pattern (1R1G1F).

Probe image

Fig II:5 Dual colour MLL LSI probe



Device slide with divisions for 24 tests



Location of probes on device coverslip

Fig II:6 Chromoprobe Multiprobe®-I

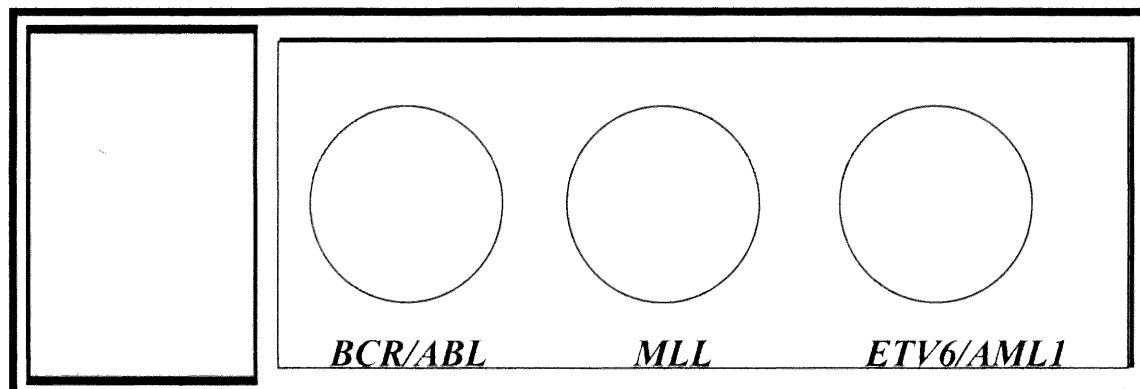


Fig II:7 Triple testing or Multiplex FISH

Chapter III

Interphase FISH for Abnormalities of *BCR/ABL*, *MLL* and *ETV6/AML1* at Diagnosis in Acute Lymphoblastic Leukaemia

III: 1 **Abstract**

The use of FISH with probes for *BCR/ABL*, *MLL*, and *ETV6/AML1* as a diagnostic tool for the accurate detection of the Ph chromosome, 11q23 abnormalities, and the t(12;21) translocation was evaluated in adult ALL.

A total of 176 adult patients were studied using interphase FISH, metaphase FISH, chromosomal analysis (CA) and PCR, for the detection of abnormalities involving *BCR* and *ABL*.

Interphase FISH detected a Ph translocation in 23 cases (13%). These included the first reported case of the insertion of *BCR* into *ABL* on an apparently normal chromosome 9 seen in an adult patient with ALL. A false positive result was found in ten cases. In sixteen of 153 (6.5%) Ph negative patients, gains of *BCR* and/or *ABL* signals were identified. Overall FISH detected alterations involving the *BCR* and or *ABL* genes in 22% of patients. Among 47 cases with combined FISH, CA and PCR it was possible to investigate discrepancies in results using these different testing methods, FISH failed to establish a correct diagnosis in 1/47 (2.1%), PCR in (0%), and CA in (0%).

In 14 selected cases, a probe with increased sensitivity for the accurate detection of *BCR/ABL* fusion was evaluated. This dual-colour LSI *BCR/ABL* ES probe is designed to produce an extra signal in Ph positive cases. It reduced the false positive rate to 0%, but did not change the rate of false negativity.

174 patients were studied for the presence of abnormalities involving the *MLL* gene, by cytogenetic analysis, interphase FISH using two probes, a single colour, and a dual-colour kit, and whole chromosome painting. Interphase FISH gave a positive

result in seven patients (4%), detecting *MLL* gene rearrangement in all five cases with a t(4;11)(q21;q23), one case with t(11;19)(q23;p13), and one case with hidden t(11;19). Loss of one *MLL* signal was seen in 2/4 cases with cytogenetic evidence of del(11)(q23). FISH revealed a false positive result in 9/119 (7.6%) cases tested with the single colour probe. The dual-colour probe showed no false positive results in 55 samples tested.

162 patients were studied using interphase FISH, whole chromosome painting, and RT-PCR for abnormalities involving *ETV6* and/or *AML1*. Interphase FISH detected *ETV6/AML1* fusion in 7 cases (4.3 %) which included two patients over the age of 40 years, and showed a 4.5% false positive rate. Losses or gains of *ETV6* and/or *AML1* were identified in 31 of 155 (20%) negative patients. In total interphase FISH detected alterations of the *ETV6* and or *AML1* genes in 23.5% of cases.

Fifteen cases were tested by both FISH and RT-PCR. Thirteen cases were negative by both methods, two cases were positive by FISH; only one of which was RT-PCR positive.

III: 2 Introduction

Abnormalities of *ABL*, and *BCR*

The Philadelphia chromosome is found in 20-35% of adults with ALL (Secker-Walker 1991; Westbrook 1992). The almost universally poor prognosis of this abnormality means that most patients are treated according to high-risk treatment protocols, and if applicable they receive an allogeneic BMT (Secker-Walker 1991; Barrett 1992; Miyamura 1992; Rieder 1993; Annino 1994; Secker-Walker 1997). For appropriate management, it is vital that these patients are diagnosed early, as they tend to have short remissions in the absence of intensive treatment modalities.

Several series have shown a higher detection rate of *BCR/ABL* fusion by molecular techniques than conventional cytogenetics (Preudhomme 1993; Radich 1994; Devaraj 1995; Rieder 1998; Campbell 1999; Mancini 2001). Due to the speed with which results can be achieved using commercially available probes on both conventionally prepared samples and cytopsin preparations (Horton 2000), interphase FISH offers potential for use in detecting this important abnormality at the time of diagnosis of ALL.

Abnormalities of *MLL*

Numerous abnormalities involving chromosome band 11q23 have been described in acute leukaemia (Mitelman 2004), the majority of these are translocations (Johansson 1998; Swansbury 1998). Translocations and other abnormalities of 11q23 involving the *MLL* gene are associated with poor outcome (Pui 1991; Rubnitz 1994; Harbott 1998). It is important to detect *MLL* gene rearrangements rapidly and accurately in adult patients presenting with ALL, since it is possible that treatment

modification, with high dose chemotherapy and bone marrow or stem cell rescue, may improve outcome (Ludwig 1998). Apart from the classical *MLL* rearrangements, rare or subtle abnormalities of *MLL* may be overlooked by CA, also due to the large number of partner genes multiplex RT-PCR is required for molecular diagnosis. Although Southern blotting has been described as the gold standard to detect variant *MLL* rearrangements. Interphase FISH analysis with probes specific for *MLL* is also independent of chromosomal or gene partners of translocations, FISH provides is a rapid and simple test, and has been recommended by other groups as a screening method for these abnormalities (Mathew 1999; Cuthbert 2000).

Abnormalities of *ETV6* and *AML1*

Since the cryptic translocation, t(12;21)(p13;q22), was described in childhood ALL by Romana et al in 1994 (Romana 1994), it has been the focus of much interest. It is found in up to 25% children with B-ALL (Shurtleff 1995; McLean 1996; Borkhardt 1997). A lower incidence has been reported in young adults, with all reported cases being less than 28 years of age (Aguar 1996; Raynaud 1996; Shih 1996; Kwong and Wong 1997; Garcia-Sanz 1999). There are indications from prospective studies that t(12;21) is associated with delayed and lower rates of relapse in childhood ALL (Harbott 1997). The low incidence of t(12;21) in adults precludes accurate evaluation of prognosis. Thus, it is vital to study this abnormality in large series of adult patients with ALL, in order to understand the biology of the disease in older patients. Since cytogenetic detection is not possible, alternative methods had to be considered. Interphase FISH screening for *ETV6/AML1* abnormalities allows the

rapid assessment of a large number of cells in samples lacking metaphases. Unlike RT-PCR, the probes will also detect the abnormality when it arises from alternative breakpoints within the *ETV6* and *AML1* loci (O'Connor 1998).

In this chapter the use of interphase FISH for the detection of structural chromosomal abnormalities of potential prognostic significance is evaluated, against cytogenetic and molecular analysis, as a diagnostic tool for their accurate identification.

III: 3 Materials and Methods:

III: 3: 1 Patients and controls:

Diagnostic samples were available from 185 adults with a diagnosis of ALL aged 15-88 years (median 28.5) presenting between February 1993 and April 2000. They included 172 patients entered to the UKALLXII trial and thirteen treated independently. In five cases the sample was inappropriate or inadequate. Of the remaining samples 180 were tested for *BCR* and *ABL* abnormalities and an evaluable result was available in 176.

Of the 174 samples successfully tested for *MLL* abnormalities; a total of 119 samples were tested using the single colour *MLL* probe, and 55 using the dual-colour *MLL* probe, and included 162 patients entered to the UKALLXII treatment trial and twelve treated independently. 162 samples were successfully tested for *ETV6/AML1* fusion, 153 were from patients entered to UKALLXII and nine from patients treated independently. Nine bone marrow samples were used as controls. (Details of patients and controls are presented in Chapter II:1)

III: 3: 2 Probes for FISH:

Cosmid probes:

Commercial probes for *BCR/ABL*, for *MLL* and *ETV6* and *AML1* were used, as described in Chapter II:4:1.

Whole chromosome paint (wcp)

Digoxigenin and biotin labeled wcps, were applied to metaphases: to identify additional chromosomal abnormalities and to confirm the partner chromosomes

involved with 11q23. Since the t(12;21) translocation is invisible, wcp 12 and 21 were used to confirm the presence of this translocation.

Alpha satellite probes:

Multiprobe-I was used for the enumeration of all 24-chromosome centromeres in patients with failed or normal cytogenetics, as described in Chapter II:4.

Individual centromeric probes were used to confirm chromosomal gains and to determine the origin of specific marker chromosomes.

III: 3: 3 Methods of analysis

Cytogenetics:

All cases were investigated by conventional chromosomal analysis as previously described in Chapter II:2.

FISH:

The same fixed cell suspensions as used for conventional chromosomal analysis were used for FISH. Slides were prepared as previously described in Chapter II:3.

All probes were hybridised according to manufacturers' instructions. (Analysis, hybridisation protocols and equipment are detailed in Chapter II).

Interphase FISH

For *BCR/ABL*, *MLL*, *ETV6/AML1* probes a minimum of 100 nuclei and up to 200 nuclei where possible were scored for each sample. For centromeric probes and the Multiprobe-I 100 nuclei were scored for each probe.

Hybridisation efficiencies and cut-off levels were established for each probe, as previously described in Chapter II:8.

Metaphase FISH:

Slides previously hybridised with specific probes were used in sequential FISH hybridisations with wcp's as previously described in Chapter II:7. This conserved scant patient material and provided maximum interpretable information on as many probes as possible in the same series of individual cells.

RT-PCR & Southern Blotting

RT-PCR for *BCR/ABL* was undertaken for patients in the UKALLXII trial, centrally by the molecular laboratory at the Hammersmith Hospital on behalf of the MRC Adult Leukaemia Working Party.

RT-PCR and Southern blotting for *ETV6/AML1* had been carried for some patients as part of a previously published study (Aguar 1996).

Results of RT-PCR testing were available for 47 of the 174 cases analysed by FISH for *BCR/ABL* and 14 of the 159 cases analysed for *ETV6/AML1*. These results were provided by Dr .J. Kaeda, Professor N.C.P.Cross & Professor J.Goldman.

In a further two cases, RT-PCR for *ETV6/AML1* was undertaken in the regional cytogenetic laboratories (Salisbury and Birmingham) and results were provided for comparison.

Immunophenotyping

Immunophenotyping was carried out by referring hospitals. Immunophenotypic subgroups of ALL were defined as follows: Null ALL (TdT + ve, CD19 + ve, CD10 - ve), common or pre-B-ALL (TdT + ve, HLADR + ve, CD19 + ve, CD10 + ve) or T-ALL (TdT + ve, CD19 - ve, CD2 + ve, CD7 + ve) or 'other'.

III: 4 Study Results

Cytogenetic analysis of the 176 cases showed a Ph translocation in 19 cases, a t(4;11)(q21;q23) translocation in five cases and a t(11;19)(q23;p13) in one case. As expected the cryptic t(12;21)(p13;q22) translocation was not identified by cytogenetic analysis alone.

Details of immunophenotype, cytogenetic analysis, sample available for FISH analysis and number of nuclei analysed are presented in Table III:1.

III: 4: 1 *BCR/ABL* fusion positive cases

Interphase FISH showed a positive result for *BCR/ABL* fusion in 23 of 176 patients. The clinical, cytogenetic and interphase FISH findings of these positive cases are presented in Table III: 2. Between 8-97% (mean $67.6 \pm 30.2\%$) of nuclei showed co-localization of *BCR/ABL* suggesting the presence of Ph translocation. FISH indicated that the breakpoint had occurred in M-BCR in ten patients and in m-BCR in the remaining 13. Interphase FISH diagnosis of *BCR/ABL* rearrangement was confirmed by chromosomal analysis (cases 1-18), metaphase FISH and/or PCR (cases 2, 5, 7, 9, 14 & 19).

Among the 18 cases with cytogenetic evidence of Ph chromosome, in five the t(9;22)(q34;q11) was the sole karyotype change (cases 1-6), additional cytogenetic findings were found in a further 10 cases, abnormalities of chromosome 7 being the most common, in five cases (9, 11-13, & 16).

In two cases the *BCR/ABL* fusion corresponded to a variant Ph translocation described as, t(2;9;22)(p1?2;q34;q11) (case 17) and t(1;9;22)(p36;q34;q11) (case 18).

Five cases had no Ph chromosome by conventional cytogenetic analysis, in three (cases 19-21) conventional cytogenetic analysis failed to produce a cytogenetic result due to poor quality metaphases, and in one (cases 22) cytogenetic analysis showed only normal metaphases, in case 23 cytogenetic analysis showed no apparent abnormality in chromosomes 9 nor 22.

Case 19 was initially reported to have a failed karyotype, with 15 normal metaphases. FISH on the same sample showed fusion signals in 80% of nuclei and on a derived chromosome 22 in five of 56 metaphases examined, with additional evidence for an m-*BCR* breakpoint. The sample was re-examined and analysis of an additional 10 metaphases revealed one with a classical Philadelphia chromosome.

In case 20 cytogenetic analysis failed, as only a small number of poor quality metaphases were available for examination. Interphase FISH showed the presence of fusion signals and the M-*BCR* breakpoint in 80% of nuclei and metaphase FISH confirmed the presence of the *BCR/ABL* fusion on derived chromosome 22 in two poor quality metaphases. Upon review a derived chromosome 22 was found suggestive of Philadelphia in one metaphase.

In case 21 hyperdiploid metaphases were observed but the poor chromosome morphology precluded accurate enumeration and analysis. Interphase FISH revealed one fusion signal in 45% of nuclei and duplication of the fusion in a further 28% of nuclei (Fig III: 1).

In case 22 the sample provided for analysis was peripheral blood. A total of 20 metaphases showed no chromosomal abnormalities, interphase FISH showed *BCR/ABL* fusion signals in 11-15% of nuclei, but in none of the metaphases.

In case 23 cytogenetic analysis of bone marrow showed a deletion of the long arm of chromosome 13. Interphase FISH showed the presence of fusion signals in over 70% of nuclei. Metaphase FISH showed both the *ABL* genes on apparently normal chromosomes 9, one copy of *BCR* on an apparently normal chromosome 22, the second copy of *BCR* was juxtaposed with the *ABL* gene on the long arm of chromosome 9 (Fig III: 2). RT/PCR confirmed the presence of *BCR/ABL* mRNA transcript. This is an example of Ph negative *BCR/ABL* positive ALL and at the time was the first case of insertion of *BCR* into *ABL* to be recognised in an adult with ALL. A similar case has since been reported (Terre 2001). Wcp redefined the del(13) as an unbalanced translocation involving chromosomes 13 and 14 producing a der(14)ins(13;14). In a small number of metaphases additional further translocations involving part of chromosome 9 were also found. Inadequate metaphases precluded the complete identification of all partner chromosomes.

Overall Ph positive patients had a higher median age (36 years), than the group as a whole (28 years), as well as a higher median WBC at $17.4 \times 10^9/l$ compared to $9.7 \times 10^9/l$.

III: 4: 2 *BCR/ABL* ES probe in cases with equivocal results

On comparing results from interphase FISH with those from conventional cytogenetics and where available PCR, a number of cases with interesting findings were seen, a number of these cases are presented in Table III.3. These included fourteen patients with low rates of *BCR/ABL* fusion of between 4.5% and 12.3%, slightly higher than the cut-off level of positivity (4.1%). Ten patients (with fusion rates of between 4.5% and 9%) with the absence of cytogenetic (in seven) or PCR

(in four) evidence of a Ph chromosome, were considered as potentially having false positive results (cases E-L). In four other patients, conventional cytogenetics showed a Ph chromosome while FISH revealed *BCR/ABL* fusion rates of between 8% and 12.3% (cases A-D). In two further cases there was cytogenetic or PCR evidence of a Ph chromosome, but FISH results were negative (cases M & N).

In order to study this patient group in more detail further testing was carried out using a more sensitive LSI *BCR/ABL* ES probe (as described in Chapter II:4).

A total of 14 cases were successfully tested with this probe. Details of the findings are given in Table III: 3. The cases included the four patients with probable true but low level positivity (cases A-D), eight of the ten cases with possible false positive FISH, where there was material remaining (cases E-L), and the two cases with possible false negative FISH (cases M & N).

The use of this ES probe distinguished between positive cases with low rates of fusion (cases A-D) and false positive results (cases E-L). In the positive cases, there was clear concordance in fusion rates using both FISH probes, with the exception of case B. In this case, cytogenetics showed only normal metaphases, interphase FISH showed a low level of *BCR/ABL* fusion, and testing by Multiprobe-I showed loss of one 9 centromere signal in a small proportion of nuclei. The absence of evaluable metaphases precluded further studies, but it is possible that in addition to the t(9;22)(q34;q11) there was further loss of chromosome 9 material including the *ASS* gene in a proportion of cells.

Although using the ES probe did not allow unambiguous identification of *BCR/ABL* fusion in the two cases with negative FISH by dual colour probe (cases M and N), it

is of note that in these two cases a small proportion of interphase cells did show the extra-signal fusions, compared to the universal absence of any extra-signal fusion signals in the eight negative cases. Overall the rate of false positive results using the dual colour probe was 10/153 (6.5%) (confirmed negative with the ES probe), and the false negative results 2/153 (1.3%), using the ES probe reduced the false positive rate to 0%, but did not change the rate of false negativity.

III: 4: 3 FISH versus cytogenetics and RT-PCR

In a number of cases, FISH, conventional cytogenetics and RT-PCR results were available for comparison. This allowed the sensitivity and accuracy of the individual techniques to be evaluated.

Results were discrepant between FISH and CA in 4/145 cases (2.75%). Interphase FISH detected three additional positive cases. FISH was negative in one case with positive cytogenetics. This case was difficult in all aspects, CA showed the majority of metaphases to be normal, only one metaphase with poor chromosome morphology was abnormal with a questionable t(9;22) and additional abnormalities (case N in Table III:3). The diagnostic sample tested negative by FISH and produced equivocal results on PCR, a second sample sent for PCR was positive. Clinically the patient relapsed rapidly despite undergoing a transplant procedure and samples at relapse showed the Ph chromosome. Interphase FISH was negative in a further case with a positive PCR, and failed cytogenetics (case M in Table III:3). It is difficult to determine whether this represents a false negative FISH or a false positive PCR result.

A total of 64/176 patients were tested for the *BCR/ABL* rearrangement by RT-PCR. Five patients demonstrated a rearrangement in m-BCR, four in M-BCR and 55 patients gave a negative result. The combination of PCR and successful CA produced discrepant results in one of 48 cases, with the detection of the Ph negative, *BCR/ABL* case (case 23 in Table III:2).

In addition to CA, complementary FISH or PCR, improved detection of Ph chromosome. However, the combination of two methods seems to be necessary for the accurate detection of cases with atypical results.

III: 4: 4 *MLL* rearrangements detected with the combination of the single colour *MLL* probe and centromere probe

In 11/119 samples tested with the single colour (*MLL*) probe, 30-75% of nuclei showed three signals suggesting the presence of *MLL* rearrangements, and in one sample a single *MLL* signal was seen in 30% of nuclei. Seven of these cases tested with chromosome 11 centromeric probe showed that the extra *MLL* signal resulted from a gain of an extra copy of chromosome 11 (to be presented later). Clinical, cytogenetic and interphase FISH details of the remaining five cases are shown in Table III: 4.

Cytogenetic analysis identified a t(4;11)(q21;q23) in three cases (24-26), The fourth case (27) metaphase FISH showed splitting of the *MLL* signal between two chromosomes, whole chromosome painting identified the presence of a translocation t(11;19)(q23;p1?). The fifth case (28) had a terminal deletion of chromosome 11 at

11q23 by cytogenetics, this was confirmed by FISH showing loss of one copy of *MLL* in 30% of nuclei.

In two further cases three *MLL* signals were seen in 7 and 7.7% of nuclei, no extra copies of chromosome 11 were seen by cytogenetic analysis. Further testing, as detailed in section III: 4: 6, confirmed these to be false positive results. Using the single colour *MLL* probe in isolation gave a false positive result in 9/119 (7.6%) cases, seven of which were due to trisomy 11, a combined approach of cytogenetics and interphase FISH with *MLL* probe and chromosome 11 centromeric probe reduced the false positive rate to 2/119 (1.7%) cases.

III: 4: 5 *MLL* rearrangement detected by the use of a dual-colour probe

In 46 of the 55 samples tested using the dual colour *MLL* probe, two *MLL* fusion signals indicative of two normal copies of chromosome 11 were seen. In these cases the number of split signals was below the cut-off point of 2.79% and ranged from 0-2.6% ($0.37 \pm 0.48\%$).

In three cases 82-94% of nuclei showed a split *MLL* signal indicating an *MLL* gene rearrangement. Cytogenetic analysis showed a t(4;11)(q21;q23) in 2 cases (cases 29 and 30), a t(11;19)(q23;p13) in one (case 31) Details of the findings in these cases are shown in Table III: 5.

III: 4: 6 Single versus Dual-colour probe for *MLL* rearrangements

Comparison of results from the two probes showed that there was a difference in the level of false positive results. The single colour probe identifies cases with trisomy 11 as positive. Thus the dual-colour probe, with a very low false-positive rate is more specific. Both probes were sufficiently sensitive to detect all cases identified as positive from cytogenetics.

For a direct comparison between the probes, 26 cases were analysed using both probes. These included nine normal control samples, six ALL samples without t(4;11) or trisomy 11 on routine cytogenetics, two with cytogenetic evidence of del(11)(q23), three with borderline increase in the number of *MLL* signals with the single-colour probe, two with hidden trisomy / tetrasomy 11 detected by Multiprobe-I, three cases with cytogenetically confirmed t(4;11)(q21;q23), and one with a hidden t(11;19). Abnormal results are presented in Table III: 6.

The dual colour probe allowed clear distinction between false positive results with three signals at low levels with the single-colour probe (cases A-C) and cases with extra copies of chromosome 11 (cases D and E) from true positive cases (cases F-I). The positive cases showed a higher proportion of positive cells with the dual colour probes compared to the single colour probe.

III: 4: 7 *ETV6/AML1* fusion positive cases

In seven cases 50-100% of nuclei showed co-localization of *ETV6* and *AML1* indicating the presence of the t(12;21) translocation. The clinical, cytogenetic and interphase FISH findings of the positive cases are presented in Table III: 7. The

positive interphase FISH results were confirmed on metaphases by the observation of the *ETV6/AML1* fusion signal on the derived chromosome 21 in all cases. This was confirmed by dual application of wcp12 and 21, showing exchange of material between these two translocated chromosomes (cases 33, 35, and 36) (case 33 in Fig III: 3) and by RT-PCR (case 38), in the other three cases with complete cytogenetic analysis (33, 35, 36) abnormalities of 12p were detected as part of a complex karyotype.

The patients ranged in age from 15-50 years and they all showed a c-ALL phenotype. The presentation white cell counts were below $10 \times 10^9/l$ in all except one patient. Two patients (32 & 35) relapsed at 11 and 24 months and did not achieve a second remission. A third (34) developed secondary acute myeloid leukaemia within 29 months of presentation. The follow-up for the remaining four patients was short, ranging from six to 27 months.

In a further seven patients, 4.8-7.2% of nuclei showed co-localization of *ETV6* and *AML1* suggesting the presence of the t(12;21)(p13;q22) translocation in a low proportion of cells. In none of the seven cases however was the fusion signal observed in metaphase cells, while RT-PCR was negative for the one case tested. In view of this lack of confirmation and the low level of fusion signals, these results were presumed to be false positive, at an incidence of 4.5% (7/155 cases).

III: 4: 8 Additional abnormalities

In addition to the positive results (*BCR/ABL* fusion, *MLL* rearrangement, or *ETV6/AML1* fusion), abnormalities involving gains or losses of signals were found in a total of 44 patients. Six of these showed abnormal signal number in addition to

the positive results. The abnormal copy of number of *MLL*, *ETV6*, and *AML1* was consistent with the cytogenetic findings of hyperdiploidy in cases 10 (Table III.2), 33 and 38 (Table III.7) and deletion of 11q23 in case 28 (Table III.4). In case 21 (Table III.2) the additional copies of *AML1* were consistent with trisomy/tetrasomy 21 in a hidden hyperdiploid clone detected by Multiprobe-I. Case 29 (Table III.5) showed unexpected loss of *ETV6*. This deletion was cytogenetically invisible.

The findings in the remaining 38 cases with abnormal numbers of one or more of *BCR*, *ABL*, *MLL*, *ETV6*, *AML1* signals are shown in (Table III: 8). In 20 cases the FISH results concurred with cytogenetics. In one case (39) with a visible 11q23 deletion, and seven cases (40-46) with visible abnormalities of 12p, there was loss of one *MLL* and one *ETV6* signal respectively. In eleven cases with extra copies of chromosome 21 (cases 45-50, 52-54), chromosome 22 (cases 49 and 51), and chromosome 11 (cases 50-52) there were concordant gains of *AML1*, *BCR*, and *MLL*, respectively, while in three cases (53-55) with very incomplete karyotypes, the extra signals observed were not unexpected given the numbers of chromosomes involved in the underlying hyperdiploidy. In cases 56-59 the extra copies of *ABL* were consistent with cytogenetic findings of a dicentric chromosome 9 or a similar chromosomal rearrangement, giving rise to duplication of 9q.

In six cases with failed or normal cytogenetics (60-65), the numbers of centromeric signals for chromosomes 9, 11, 12, 13/21, and 14/22, successfully defined by Multiprobe-I, agreed with those for *ABL*, *MLL*, *ETV6*, *AML1* and *BCR*. (These cases are discussed in further detail in chapter IV). Thus findings from the sequence specific probes can be indicative of numerical chromosomal changes.

the length of an abnormal marker, which subsequently proved to be composed entirely of chromosome 21 material (this case is detailed further in chapter V)(Fig III:4).

In summary, 38 (27.5%) ALL patients who were negative for *BCR/ABL* fusion, *MLL* rearrangement and *ETV6/AML1* fusion did not show the expected two copies of the tested genes, although the abnormalities corresponded to the cytogenetic result in half the cases; the other 17 cases only came to light by the particular combination of techniques employed.

III: 5 Discussion

Testing for *BCR/ABL*

Extensive FISH based study has been carried out to detect cases of Ph positive ALL in a large series of adult patients. Initial testing with dual colour probes applied to interphase nuclei preparation identified the *BCR/ABL* rearrangement in all but one of the 19 patients shown to have a Ph translocation by cytogenetics. An additional five cases, with hidden Ph were identified in which the Ph chromosome was not seen by conventional karyotyping due to the poor sample quality, low level of the abnormality, or variant Ph translocation. The probe distinguishes between M-*BCR* and m-*BCR* breakpoints, and in the six cases with both FISH and RT-PCR results there was almost complete concordance in the results obtained.

Ten patients tested with the dual-colour *BCR/ABL* probe showed fusion signals at a low level, suggesting a Ph translocation in a small proportion of cells. Evidence of a true Ph translocation was not achieved from cytogenetics, or PCR in a number of these cases, and further testing with the sensitive ES probe kit confirmed that the low fusion rate was due to the chance co-localisation of *BCR* and *ABL* in these nuclei, rather than a genuine translocation. In three of these ten cases a hyperdiploid clone was identified with extra copies of *ABL* and/or *BCR*. The false fusion signals were identified within these hyperdiploid nuclei only. This type of association has been previously described using the same probe (Rieder 1998). It is probable that with the increased numbers of signals increases the likelihood that a chance co-localisation will occur. Overall the false positive rate with this probe in this series was 10/176 (5.6%).

The use of an *ABL* probe that extends to and includes sequences from the *ASS* gene, beyond the breakpoint cluster region of *ABL*, increases the sensitivity of the FISH test. Dual-fusion or triple signal probes have been applied successfully in diagnosis and in the study of minimal residual disease in CML (Sinclair 1997; Buno 1998). These probes have not been used extensively in ALL but the same principle applies. In this series this probe was used in cases with a suspected false positive result. Comparisons were made with normal controls, and cases confirmed to have a low level Ph positive clone, as negative and positive controls respectively. In the normal and the false positive cases, the rate of fusion was consistently found to be zero, whereas true Ph positive cases the detection of a fusion was accurate even at a level of only 5%. An interesting observation was that in the “false-positive” cases a population of cells with closely aligned *BCR* and *ABL* signals within their nuclei was consistently observed, this may be due to the structural orientation of the sequences of these genes preferentially lying in close proximity within the nuclei.

False negative FISH results were obtained in two cases with cytogenetic or PCR evidence of a Ph chromosome. The incidence of fusion signals was 1.5% and 3.75% in these cases. This phenomenon of low level values, within the normal range for controls, has previously been described (Dewald 1993). It is likely to be related to the quality of the sample in this study, and may be caused by the gradual depletion of positive cells from the regularly re-used cell suspension. In this study, material used for FISH were samples provided after completion of cytogenetic analysis in other laboratories. In one of the positive cases the rate of positivity by FISH dropped from 15% to 11% to 5% in successive sampling of the same fixed cell pellet.

Cytogenetic analysis revealed only one Ph positive metaphase from 20 examined, implying low level of positivity, and in the second case examination of the initial sample by conventional cytogenetics failed, FISH for *BCR/ABL* fusion on this sample was negative, the sample taken at the same time for RT-PCR was inconclusive, a repeat sample sent for RT-PCR only was positive, this implying that initial sampling quality was the cause for false negativity of tests.

In general this study has confirmed the efficiency of dual-colour probes for interphase FISH screening of Ph positive ALL. Cases with low levels of positive nuclei can be verified by testing with the ES probe. Testing with ES probe reduces the rate of false positive results but not necessarily the rate of false negatives.

In the five cases with FISH evidence of a *BCR/ABL* rearrangement without a Ph chromosome a number of events explained the difficulties of cytogenetic analysis. In one case this was due to the low proportion of abnormal cells in peripheral blood. Several previous studies have clearly shown the superiority of bone marrow over blood samples in the initial diagnosis of ALL, especially for karyotyping (Secker-Walker 1997). In the second case, despite the high proportion of abnormal cells found by FISH, there appeared to have been a selective growth advantage of the non-malignant cells, since lower proportions of Ph positive metaphases were observed than would be expected from the interphase FISH results.

The association of hyperdiploidy with the presence of a Ph chromosome is not uncommon in adult ALL. It is recognized that chromosome morphology may be poor in cases with more than 60 chromosomes, making identification of structural abnormalities difficult. In one of the five cases described here this was so. However

this case serves to illustrate the powers of FISH, in defining abnormalities in metaphases of poor quality.

In only one of the five *BCR/ABL* positive Ph negative cases was the Ph chromosome truly absent. The fusion arose from the insertion of BCR into ABL on the apparently normal chromosome 9. This is a well-described finding in CML, but rarely reported in ALL and is believed to be the result of a two-step event. In the first step a classical Ph translocation takes place and this is followed by a second translocation between chromosome 9 and 22 that results in the two chromosomes appearing morphologically normal but with the fused *BCR/ABL* genes now located on 9q (Nacheva 1994).

In five *BCR/ABL* positive cases further studies utilising wcp to define chromosomal translocations, provided evidence of involvement of other chromosomes with an impact on prognosis (Rieder 1996) these included chromosomes 7, 9p and the presence of hidden hyperdiploidy in association with the Ph chromosome in two cases.

In addition to detection of *BCR/ABL* rearrangements the probe identified numerical abnormalities by chance findings of abnormal copy number of *BCR* and/or *ABL* in 17 cases. In three of these case the finding pointed to the presence of a hidden hyperdiploid clone, later confirmed by Multiprobe-I system. In a further three cases additional signals were not associated with numerical changes, in these cases structural abnormalities involving chromosome 9 and/or 22 could not be ruled out.

This study provides strong support for the important role of FISH in the accurate diagnosis of Ph positive ALL. The combination of interphase and metaphase FISH

with a variety of FISH probes allows the detection of rare cases with variant translocation, the accurate identification of BCR breakpoints, provides information on proportions of Ph positive and Ph negative cells at presentation and gives a more complete picture of associated abnormalities which may be related to prognosis, which provides vital information for patient management, as well as scientists studying the biology of this disease.

Testing for *MLL*

In this study two commercially available FISH probes, one single and one dual-colour, were assessed for detection of *MLL* rearrangements in a large population of adults with ALL. Comparison was made between the two probes and FISH results were evaluated against cytogenetic results. Both probes proved to be highly efficient in the detection of *MLL* rearrangement in cases with cytogenetically visible translocations involving 11q23, as well as one case with a hidden t(11;19).

In agreement with previous reports, the five cases with t(4;11)(q21;q23) in this series showed very high presentation WBC (mean $191 \times 10^9/l$) compared to the negative cases, and only one expressed the CD10 (common-ALL antigen).

In this series there were also four cases with cytogenetic evidence of 11q23 deletions. FISH with both probes showed *MLL* was deleted in two of these. *MLL* was retained in the other two cases, indicating a breakpoint outside 11q23. The distinction between these two groups is of clinical importance, as it appears that patients with 11q23 deletions involving the *MLL* gene do less favourably than those without *MLL* gene loss (Harbott 1998).

The false positive rate was lower with the dual-colour probe, 0/55 compared to 9/119 (7.6%) for the single colour probe. This confirmed the higher specificity of the dual-colour probe. The extent of the dual-colour probe, spanning the *MLL* gene beyond the breakpoint cluster region (bcr) and flanking sequences, enables the dual-colour probe to be able to detect the rare cases with breakpoints 3' of the gene, which would not be detected by the single colour probe, covering only the bcr region. Since the dual-colour probe relies on the separation of different coloured signals, rather than number of signals, it is able to detect unbalanced translocations, which would appear as normal with the single colour probe.

An observation made on examining four cases with t(4;11) or t(11;19) was that a higher proportion of positive nuclei was found with the dual colour than with the single colour probe. This may reflect differing hybridization efficiencies between the probes, or be due to difficulties in scoring extra signals. The presence of two separate signals in different colours is easier to count with confidence.

FISH is the method of choice for the rapid screening of acute leukaemia cells for the presence of rearrangements of the *MLL* gene as it detects all translocation partners, it is hoped that with the continued introduction of more sophisticated FISH probes for testing, the need for supporting methods of analysis should be reduced.

Testing for *ETV6/AML*

This study is the first to report of the use of interphase FISH for the identification of *ETV6/AML1* fusion in a series of adult patients. It also represents the largest series of adult patients in which the incidence of the translocation has been studied. The technique has been used for childhood ALL series, in which the results differ

strikingly from this one in the much higher incidence of positive cases (Ameye 2000). RT-PCR, was used in previously reported series of adult patients (Aguiar 1996; Raynaud 1996; Shih 1996; Kwong and Wong 1997; Garcia-Sanz 1999).

Seven positive cases were found among 162 adult ALL patients, an incidence of 4.3%. If known non-B ALL cases are excluded the incidence in our study is 5.1% (Jabber Al-Obaidi 2002). This figure is a little higher than those reported for other adult series (0-3.3%), which may reflect the larger size of our sample, or result from a real geographical variation in incidence (Garcia-Sanz 1999). The most likely explanation however, is that FISH may identify cases with alternative breakpoints in the gene which are not detected by RT-PCR (O'Connor 1998). In our series RT-PCR was performed on 16 cases, but one of these proved to be positive by FISH and negative by RT-PCR. Previously described *ETV6/AML1* fusion positive adults were aged between 15 and 27 years. Two of our positive cases were older and at 45 and 50 years respectively, are the oldest *ETV6/AML1* fusion positive cases reported. Similar to findings in childhood cases, *ETV6/AML1* fusion was associated with a low white blood count, a common ALL phenotype, cytogenetically identifiable 12p abnormalities and in two cases a near triploid/tetraploid clonal karyotype (Romana 1995; Romana 1996; Raimondi 1999).

The deletion of the second *ETV6* allele, which has been reported to occur frequently in children in association with the fusion gene, was only observed in two of the positive adults. Although the occurrence of intragenic deletions or point mutations undetectable by FISH techniques cannot be ruled out, outcome could be in some way related to the retention of this second allele.

Due to the infrequency of the t(12;21) translocation in adults, its influence on prognosis has yet to be resolved. In this series, two of the *ETV6/AML1* fusion positive patients relapsed. One with a complex karyotype had retained the second copy of the *ETV6* allele in the abnormal cells at both diagnosis and relapse. In another, cytogenetics had failed, while interphase FISH showed loss of the second copy of the *ETV6* gene. A third patient developed secondary acute myeloblastic leukaemia within 29 months of presentation. Firm conclusions on the influence of this translocation on outcome await a longer follow-up and larger numbers of patients. However it appears unlikely that the t(12;21) translocation is associated with a uniformly better outcome in adults.

The commercial *AML1* probe used spans the breakpoint on chromosome 21 and is designed to give an extra signal on the derived chromosome 12 in positive cases. In this study the additional signal was only clearly visible in three of the positive cases and then in only some of the positive nuclei. Care must therefore be taken in relying on the extra signal for confirmation of positivity. A possible explanation is that there has been a partial deletion of the *AML1* gene involved in the *AML1/ETV6* fusion. In our laboratory, specific probes to the exons of the *AML1* gene were used together with wcps for chromosomes 12 and 21 on some positive childhood cases. They showed that the 21 material translocated to the short arm of chromosome 12 did not always include *AML1* exons, indicating that they had been deleted (GR Jalali, personal communication). This interpretation is supported by similar results obtained with probes to the *MLL* gene (Cherif 1994).

The sensitivity of dual-colour interphase FISH is limited by the occurrence of false positive results. Studies using similar probes for *BCR/ABL* fusion showed that hyperdiploidy (Rieder 1998) or the small-condensed nuclei characteristic of T-ALL (Chase 1997) may produce higher rates of false positivity. In our series only one each of the 16 cases with hyperdiploidy and the 25 cases with T-ALL had slightly raised fusion rates. However in all the false positive cases, the proportion of fusion signal positive nuclei was far lower than in the confirmed t(12;21) cases. The cut-off points for positivity need to be determined by each investigator independently, but it appears that higher cut-off levels for a false positive result are required in the presence of extra *ETV6* and or *AML1* signals, or very small blast nuclei. In cases with a low level positive result (<10%), the t(12;21) status should be verified by chromosome painting and/or RT-PCR to confirm a positive finding.

Thirty-one cases negative for *ETV6/AML1* fusion did not show expected normal pattern of two signals for each of the genes in interphase. There was, however, a close agreement in the majority of these cases between the interphase FISH results and those obtained by cytogenetics or by Multiprobe-I system. The case in which only one cytogenetic population appeared to be represented at interphase could reflect a real chromosomal difference between cells in division and in interphase, a feature which we have noted in our laboratory in some cases of hyperdiploidy.

In four cases the result obtained with the *ETV6* probe was the only indication that there had been deletion of the gene. This reflects the common association between ALL and abnormalities of 12p (Baccichet and Sinnett 1997). This we have also

shown in our laboratory, 3/30 cases of ALL negative for *ETV6/AML1* fusion showed deletions of *ETV6*, which were not suspected from the cytogenetics. Extra copies of chromosome 21 are also a recurrent feature of ALL (Moorman 1996). The finding of additional copies of *AML1* was the first clue to the presence of hidden hyperdiploidy, confirmed by Multiprobe-I in seven patients. In three other cases discordant results between cytogenetics and interphase FISH led to the discovery of *AML1* amplification; which has been described in both childhood and adult ALL (Le Coniat 1995; Najfeld 1998; Niini 2000). In one of these cases which has been published (Harewood 2003) the extra signals were found on a quadruplicated chromosome 21, a finding previously reported in a child (Baialardo 1996).

In the light of our results, we conclude that dual-colour *ETV6/AML1* interphase FISH is a simple, efficient and relatively quick method of screening for the t(12;21) translocation among adult patients with ALL. The translocation appears to occur over a larger age range than previously suspected and may be more prognostically heterogeneous in adults than in children. An added advantage in using these probes is in the extra genetic information they provide about patients who do not have the fusion gene.

The reliability of the technique is underlined by the remarkable agreement between the results it produced and those obtained with the other methods employed. Although occasional 'false positives' do occur, they are easily distinguishable from true positivity by the small proportions of cells they involve. Thus confidence can be placed in the exclusive use of interphase FISH for determining *ETV6/AML1* fusion.

Table III.1 Patients grouped by successful FISH analysis.

	FISH STUDIES								SAMPLE			PATIENTS			IMMUNO					CC		
	Total	D ⁰	M ⁰	F	L	AN	≥200n	<200n	BM	PB	M+B	Tr	NT	C	PreB	T	O	N	U	A	N	F
<i>BCR</i>	193	0	4	5	8	176	138	38	158	12	6	163	13	81	35	27	7	2	24	104	37	35
<i>MLL</i>	193	4	4	5	6	174	157	17	157	11	6	162	12	81	34	27	9	1	22	104	36	34
<i>ETV6</i>	193	6	4	15	6	162	124	38	145	11	6	154	8	73	34	25	9	1	20	94	36	32
<i>MP</i>	83	5	27	3	0	48			39	7	2	43	5	19	7	12	2	1	7		27	21
<i>BCR; BCR/ABL</i>				<i>ETV6; ETV6//AML1</i>				<i>MP: Multiprobe-I</i>				<i>D⁰: Not done</i>				<i>M⁰: Inadequate material</i>						
F: Fail				L tested elsewhere				AN: analysed in study				≥200n: at least 200 nuclei analysed										
<200n: less than 200 nuclei analysed				M: Bone marrow				B: Peripheral blood				M+B: Bone marrow and peripheral blood										
Tr: patient within UKALLXII trial				NT: Patient treated independently				C: CD10+CommonALL				T: T-ALL				O: Other						
N: null ALL				U: No immunophenotype available				A: Abnormal clone identified				N: 20 normal metaphases analysed										

Table III.2 Ph positive cases

Case	UIN	Clinical Features				Treatment and outcome		FISH Results				BCR/ABL PCR	G-Banded Karyotype
		Age	Sex	WBC	Immuno	BMT	Outcome	no of signals (% of abnormal nuclei)		MLL	ETV6	AML1	
1	143	43	M	12	c-ALL		EFS 8mo	F 97	BCR- B m-BCR				46,XY,t(9;22)(q34;q11)[7]/ 46,XY[5]
2	104	37	M	4.6	c-ALL		BM relapse 5mo	12.3	m-BCR			e1a2	46,XY,t(9;22)(q34;q11)[7]/ 46,XY[5]
3	146	42	M	19.4	c-ALL		EFS 8mo	31	M-BCR				46,XY,t(9;22)(q34;q11)[12]/ 46,XY[6]
4	98	20	M	330	Pre-B		BM Relapse 7 mo	78	m-BCR				46,XY,t(9;22)(q34;q11)[18]/ 46,XY[1]
5	132	47	F	2	c-ALL		Died in CR 3 mo	91	m-BCR			e1a2	46,XX,t(9;22)(q34;p11)[30]
6	176	50	F					80	M-BCR				46,,XX,t(9;22)(q34 ;q11)[27]/ 46,XX[6]
7	74	41	F	2.2	Other ALL	Allo1 st CR	EFS 29 mo	8	m-BCR			b3a2	45,XX,t(9;22)(q34;q11), add(3)(q27),-10,+mar[5]/ 46,XX[2]
8	129	23	F	2.1	c-ALL		EFS 16mo	12	m-BCR				46,XX,t(9;22)(q34;q11), add(14)(q12)[4]/ 46,XX[6]
9	88	26	M	6.5	Pre-B	Allo1 st CR	BM Relapse 15 mo	50	m-BCR			e1a2	45,XY,-7,t(9;22)(q34;q11),10, +mar[9]
10	155	22	F	2.9	c-ALL		EFS 5mo	70	M-BCR	3 (28%)	3 (28%)	4 (28%)	57,XX,+X,t(9;22)(q34;q11), +13,+15,+15,+17,+18,+19, +19,?del(19)(p13p13),+21,+21, +der(22)t(9;22)(q34;q11)[cp8]/ 70,dem,+X,+1,+2,+4,+5,+6, +6,+8,+10,+11,+12,+14,+14, -15,+17,-21,+22/[cp8]72,idem,+1, +2,+2,+4,+5,+6,+8,+8,+ der(9)t(9;22)(q34;q11),+10, +11,+12,+13+14,- 15,+18,+20[cp8] 46,XX[24]

11	118	34	F	28.2	c-ALL	MUD 2 nd CR	BM Relapse 8 mo	75	m-BCR						47,XX,t(9;22)(q34;q11), +i(17)(q10),inc[6]/idem,-7, der22 t(7;22)(q11.2;q11)t(9;22),inc[27]/ 46,XX[6]
12	117	35	M	8		Allo 1 st CR	EFS 17 mo	85	m-BCR	1 (87%)					46,XY,del(3)(p13),del(7)(p13), t(9;22)(q34;q11),del(11)(q13)[8]/ 46,XY[23]
13	148	41	F	64.7			EFS 8 mo	86	m-BCR						44,X,-X.add(1)(p36.3), add(3)(p2.3),-7,-9, t(9;22)(q34;q11),+mar[15]/ 45,X,- X,idem,+der(22)t(9;22)[3]/ 46,XX[2]
14	31	46	M	277	c-ALL	MUD 2 nd CR	BM Relapse 4 mo	87	M-BCR			b3a2			46,XY,t(9;22)(q34;q11)[3]/ 45,idem,del(9)(p22), dic(20;22)(q11;p11)[27]
15	125	24	F	43.3	Pre-B		BM Relapse 7 mo	88	m-BCR						45,XX,der(9)t(9;22)(q34;q11) der(9;22)(q10;q10),der(22)t(9;22)
16	108	33	F	24.7	c-ALL	Allo 1 st CR	EFS 19 mo	94	M-BCR						44,XX,-7,der(9)t(9;22)(q34;q11), add(9)(p1)
17	91	21	M	38	T-ALL		BM Relapse 10 mo	90	M-BCR						46,XY,t(2;9;22)(p1?2;q34;q11), -5,del(11)(q ?14),+mar[15]/ 46,XY[3]
18	113	43	F	350	c-ALL	MUD 1 st CR	Died in CR 14 m	93	M-BCR						46,XY,t(1;9;22)(p36;q34;q11)[7] 46,XX[1]
19	71	41	F	4.6		Allo 1 st CR	Died in CR 6mo	80	m-BCR			e1a2			Fail 46,XX[15]
20	175	88	F					80	M-BCR						Fail
21	174	29	M					53	m-BCR	2	3(11%) 4(32%)				Fail
22	39	55	M	17.4			Died in CR 7 mo	11-15	M-BCR						46,XY[30]
23	144	26	M	152.5			BM Relapse 6 mo	70	M-BCR						46,XY,del(13)(q12q14)[20]
Immuno:Immunophenotype				Allo: Allogeneic transplant		CR: Complete remission		BMT: Bone marrow transplant		MUD: Matched unrelated transplant					
EFS: Event free survival in months				BM: Bone marrow		F: BCR/ABL fusion signal		BCR-B: BCR Breakpoint		MLL: copy number of MLL signals					
				ETV6: copy number of ETV6				AML1: copy number of AML1							

Table III.3 Vysis *BCR/ABL* LS ES probe.

Case	UIN	LS probe FISH		ES probe FISH		PCR	G-Banded karyotype
		F%	BCR Breakpoint	F% No ES	F% With ES		
A	74	8	M-BCR	5	10	b3a2	45,XX,t(9;22)(q34;q11),add(3)(q27),-10,+mar[5]/ 46,XX[2]
B	39	11	M-BCR	1.5	4.5		46 XY[30]
C	129	12	m-BCR	1.4	11		46,XX,t(9;22)(q34;q11),add(14)(q12)[4] /46,XX[6]
D	104	12.3	m-BCR	1.5	6.5	e1a2	46,XY,t(9;22)(q34;q11)[7]/ 46,XY[5]
E	136	4.6		4	0	Neg	46,XY,del(9)(p21)[12]/46,XY[8]
F	11	4.7		5	0	NS	51,XY,+21,+mar1,+mar2,+mar3,+dmin,inc(cp4)
G	56	5		4	0	Neg	46,XY,-17,+mar[3]/47,idem,+mar2[5]/46,XY[3]
H	85	5		4	0	Neg	Fail 46,XY[13]
I	133	5		3.5	0		46,Y,t(X;10)(?q11;?p11)[6]/46,idem,del(6)(q21)[1]/45,idem,der(17)t(17;21)(?p11;q11),-21[3]/ 45,idem,der(17)t(17;21)(?p11;q11),-21,del(6)(q?) [2] 46,XY[3]
J	167	5		6	0	NS	Fail
K	84	5.5		6	0		664-180 ,inc[6]/46,XY[4]
L	147	6.6		4.5	0	Neg	46,XY[40]
M	103	1.5		2.1	0.8	e1a2	Fail
N	53	3.75		4	0.6	b2a2	45,XX,-7,?t(9;22)(q34;q11),?der(16)t(1;16),+?der(16)t(1;16),-18(1) 46,XX(19)
		ES:extra signal		F:BCR/ABL fusion signal		Neg: Negative	
						NS: No sample	

Table III.4 Cases with abnormal findings by single colour *MLL* probe

		Clinical Features				Treatment and outcome		FISH Results	G-Banded Karyotype
		Sex	Age	WBC	Immuno	BMT	Outcome in months	MLL CN	
24	135	F	50	15.1	Pre-B	No	EFS 10 mo	3 (50%)	46,XX,t(4;11)(q21;q23)[5]/47,idem,+X[4]/48,idem,+X,+der(4)t(4;11)[2]/46,XX[4]
25	66	F	35	132	Pre-B	No	BM relapse 7 mo	3 (57%)	46,XX,t(4;11)(q21;q23),i(7)(q10),del(12)(p13)[16]/46,XX[1]
26	34	M	30	192	Pre-B	MUD 1 st CR	EFS 48 mo	3 (39%)	46,XY,t(4;11)(q21;q23)[12]/46,XY[5]
27	169	M	19					3 (50%)	46,XY,der(7)9q210[3]/46,XY,add(7)(q21),del(19)(p11p13)[15]/46,XY[2]
28	105	F	32	78	c-ALL	No	Died in CR 2 mo	1 (32%)	48,XX,+X,+5,i(6)(p10),+7,-10,del(11)(q23),der(14)t(14;?)(q23:?),-15,-17,+2mar[18]/46,XX[2]
		BM: Bone marrow EFS: Event free survival				BMT: Bone marrow transplant <i>MLL</i> CN: Number of copies of <i>MLL</i> signal		CR: Complete remission NA: Not applicable	

Table III.5 Cases with abnormal findings by dual-colour *MLL* probe

Case	UIN	Clinical Features				Treatment and outcome		FISH results		G-Banded Karyotype
		Age	Sex	WBC	Immuno	BMT	Outcome	<i>MLL</i> SP	<i>ETV6</i>	
29	161	24	F	191	cALL	No	EFS 3 mo	82%	1(88%)	51,XX,+X,t(4;11)(q21;q23),+der(4)t(4;11),+6,+7,+13[4]/46,XX[11]
30	156	16	F	255	Pre-B	No	EFS 5 mo	94%		46,XX,t(4;11)(q21;q23)[4]/46,idem,add(14)(q?32)-18,+mar[3]/46,XX[1]
31	140	46	M	930	Other	Allo 1 st CR	Died in CR 5 mo	94%		46,XY,t(11;19)(q23;p13)[10]
		Allo: Allogeneic transplant EFS: Event free survival				BM: Bone marrow <i>MLL</i> SP: % of Split <i>MLL</i> signals		BMT: Bone marrow transplant <i>ETV6</i> : %of nuclei showing single <i>ETV6</i> signal		CR: Complete remission

Table III.6 Single colour versus dual-colour *MLL* probe

Case		Interphase FISH Findings										
		Single colour probe					Dual-colour probe				Multiprobe®-I	
		1S	2S	3S	4S	Split	1FS	2FS	3FS	4FS	Overall	11
A	139	0	95	5	0	0	0	97.5	1.5	1	NA	48,XX,+8,del(9)(p?21),+?14[13] 46,XX[2]
B	138	0	92.6	7.3	0	0	0	100	0	0	NA	47,XY,+X[30]
C	175	0	92.6	7.3	0	0	2	96	2	0	NM	Fail
D	45	0	53.5	63.3	0	0	0	53	47	0	Fail	3 Fail
E	87	0	91.5	0	8.5	1	0.5	82	3.5	13	Tt	4 46,XY,del(6)(q12)[10]
F	135	0	82	18	0	64	0	36	0	0	NA	46,XX,t(4;11)(q21;q23)[5]/47,idem, +X[4]/48,idem,+X,+der(4)t(4;11)[2]/ 46,XX[4]
G	66	0	43	57	0	87	0	27	0	0	NA	46,XX,t(4;11)(q21;q23),i(7)(q10), del(12)(p13)[16]/ 46,XX[1]
H	34	0	61	39	0	93	0	7	0	0	NA	46,XY,t(4;11)(q21;q23)[12]/ 46,XY[5]
I	169	0	50	50	0	96	0	4	0	0	NA	46,XY,add(7)(q21)[3]/46,XY, add(7)(q21),del(19)(p11p13)[15]/ 46,XY[2]
NA: Not applicable		NM: No material					S: signal		FS: Fusion signal		Tt: Near triploidy (>70 chromosome)	

Table III.7 t(12;21) positive cases.

Case	UIN	Clinical Features				Outcome (months)		FS	Interphase FISH results					G-Banded Karyotype
		Age	Sex	WBC	Immuno	OS	EFS		<i>ETV6</i>	<i>AML1</i>	<i>MLL</i>	<i>BCR</i>	<i>ABL</i>	
32	44	15	M	3.2	cALL	24	31	1(79%)	0	1				Fail 46,XY[12]
33	131	15	M	3	cALL	15	15	1(50%)	0	3	4 (50%)	3 (50%)	4 (50%)	90,XXYY,-5,-9,-12,del(12)(p11), del(12)(p11,+13[cp15]/ 46,XY[5]
34	26	16	F	8.3	cALL	29	29	1(64%)	1	1				Fail 46,XX[15]
35	100	25	F	5.1	cALL	11	18	1(67%)	1	1				45,X,-X,add(3)(q12),del(6)(q?16), add(9)(q?),add(12)(p11)[18]/ 45,idem,-del(6)(q?16), der(6)del(6q?16)rdup(6)(pter-q12)[2]
36	82	28	M	3.1	cALL	27	27	1(82%)	1	1				47,XY,add(8)(p1?),?add(9)(p?),del(9)(p ?), add(12)(p?),+21[13]/46,idem,-Y[11] 46,XY[3]
37	151	43	M	240	cALL	6	6	1(94%)	1	3				Fail 46,XY[9]
38	145	50	M	9.7	cALL	9	9	1(33%) 2(51%)	3	3-4	3-4	4 (90%)	3 (90%)	70,inc[33]/ 46,XY[3]
Imm Immunophenotype OS overall survival in months <i>MLL</i> : Number of copies of <i>MLL</i>						EFS Event free survival in months <i>ETV6</i> : Number of copies of <i>ETV6</i> <i>BCR</i> : Number of copies of <i>BCR</i>				FS Fusion signals: number of signals (% of abnormal cells) <i>AML1</i> :Number of copies of <i>AML1</i> <i>ABL</i> : Number of copies of <i>ABL</i>				

Table III.8 Abnormal *BCR*, *ABL*, *MLL*, *ETV6*, *AML1* copy number

		Interphase FISH						G-banded karyotype					
		<i>BCR</i>	<i>ABL</i>	<i>MLL</i>	<i>ETV6</i>	<i>AML1</i>	M-I	Individual centromeres					
		<i>R</i>					Overall	9	11	12	13/ 21	14/ 22	
39	105	2	2	1	2	2			NA				48,XX,+X,+5,i(6)(p10),+7,-10,del(11)(q23), der(14)t(14;?)(q23;?),-15,-17,+2mar[18]/46,XX[2]
40	96	2	2	2	1(96%)	2			NA				46,XY,del(12)(p?11~p13),del(13)(q12q14)[20]/46,XY[1]
41	37	2	2	2	1(60%)	2			NA				46,XY,del(5)(q13q35),add(10)(p13~15),del(12)(p11)[6]/ 46,XY[17]
42	116	2	2	2	1(19%)	2			NA				45,XX,del(6)(q13q21),-7,add(12)(p1?)[12]/46,XX[26]
43	75	2	2	2	1(68%)	2			NA				45,XX,dic(9;12)(p1?;p1?),der(19) t(1;19)(q23;p13)[2]/46,XX[4]
44	154	2	2	2	1(68%)	2			NA				45,XX,dic(9;12)(p1?;p1?),del(17)(p1?)[20]
45	168	2	2	2	1(97%)	3(100%)			NA				47,XX,t(8;14)(q1?1;q32),del(12)(p12p13),+21c
46	43	2	2	2	1(56%)	3(56%)			NA				47,XY,del(12)(p?),+21[12]/46,XY[14]
47	112	2	2	2	2	3(80%)			NA				46-47,XX,del(6)(q21q25),del(13)(q14q22),+21,inc[cp]
48	80	2	2	2	2	4(42%)			NA				56,XY,+X,+Y,+4,+8,+10,+14,+17,+18,+21,+21[11]/ 46,XY[9]
49	89		2	2	2	4(55%)			NA				61,XY,+X,+Y,+4,+5,+6,+8,+10,+14,+17,+18,+18,+20, +21,+21,+22[18]/46,XY[7]
50	60	2	2	3	3(33%)	3(33%)			NA				54-61,XY,+X,+Y,+1,add(1)(p13),+add(1),+4,+7, der(9)t(8;9)(q?13;p?13),+11,+12,+13,+14,add(14)(q32), +17,+19,+20,+21,-22,-22,+mars,inc[cp15]/46,XY[1]
51	158	4	2	3	2	2			NA				59-60,XX,+X,+1,+5,+6,+8,+8,+10,+14,+18,+18,+19,+20, i(21)(q10),+22[cp4]/46,XX[1]
52	173	2	2	3	2	2			NA				52,inc
53	30	2	2	2	2	4(83%)			NA				53,+4,inc[cp4]/46,XX[2]
54	50	4	3	3-4	3(44%) 4(48%)	4(86%)			NA				85-87,XXYY,+mar,inc[4]/46,XY[2]
55	84	4	3	3-4	1(17%) 3(7%)	3(19%) 4(58%)			NA				64-180,inc[6]/46,XY[4]
56	15	2	3	2	2	2			NA				46,XY,der(9')t(9';9)(p22;q22),der(19)t(1;19)(q23;p13)[4]

57	68	2	3	2	2	2		NA									46,XY,i(9)(q10),i(17)(q10)[15]
58	123	2	3	2	2	2		NA									45,X,-Y,add(3)(q21q23),del(6)(q1?3q2?1),i(9)(q10), add(14)(q32),i(18)(q10)[5]/45,idem,add(12)(p13),+18,- i(18)(q10)[2]/46,XY[2]
59	164	2	3	2	2	2		NA									46,XX,add(2)(p),add(3)(p),-5,-6,add(7)(q),i(9)(q10), -13,add(14),der(14;17),+4xmar
60	19	2	2	2	2	1(21%)	Ho	2	3								Fail 46,XY[15]
61	120	2	2	2	2	3(35%)	HeL	2	5								Fail
62	110	2	2	2	2	3(12%)	HeL	2	5								Fail
63	85	2	2	2	2	3(45%)	HeH	2	5								Fail 46,XY[13]
64	21	3	4	3	2	3(10%) 4(43%)	HeH	3	3	2	5/6	6					46,XX[20]
65	12	3	2	3	3(41%)	4(41%)	Tr	2	4	F	6	5					Fail
66	171	2	2	2	2	4(72%)	HeH			2	5						Fail
67	87	4	4	4	4(13%)	4(13%)	Tt	4		4	6	8					46,XY,del(6)(q12)[10]
68	124	2	2	2	1(18%)	2	NA										46,XX,?del(9)(q21q33)[4]/46,XX[6]
69	93	2	1	2	2	2	NM										46,XX[20]
70	126	3	2	2	1(15%)	2	NM										Fail
71	40	2	2	2	0(85%)	2	NA										46,XY,inc[68]
72	72	3	2	2	2	2	NM										Fail
73	45	1	1	3	2	2	NM		3								Fail
74	159	2	2	2	2	5(77%)	HeH			2	4						46,XY[20]
75	11	2	2	2	6(33%) 8(31%)	2	NA										50,XY,+21,+mar1,+mar2,+mar3,+1dmin,inc[cp4]
76	101	2	2	2	2	4(55%)	NA										46,XY,del(7)(p1?5),t(8;22)(q1?1;q13),dup(21)(q)

ABL : copies of ABL signal

NA: Not applicable

Tt: (near triploidy >70 chromosomes)

13/21: Number of copies of DZ131/DZ211

HeL: Low Hyperdiploid (47-50 chromosomes)

BCR : copies of BCR signal

NM: No material

ETV6: Number of copies of ETV6

NA: Not applicable

HeH: High Hyperdiploid (51-65 chromosomes)

9: number of copies of DZ91

HeH: High hyperdiploidy (51-60 chromosomes)

AML1: Number of copies of AML1

NM: Insufficient material

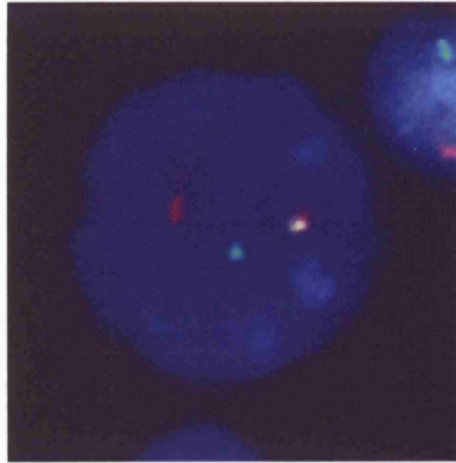
Tr: Near Triploid (66-80 chromosomes)

14/22: number of copies of DZ141/DZ221

12: Number of copies of DZ121

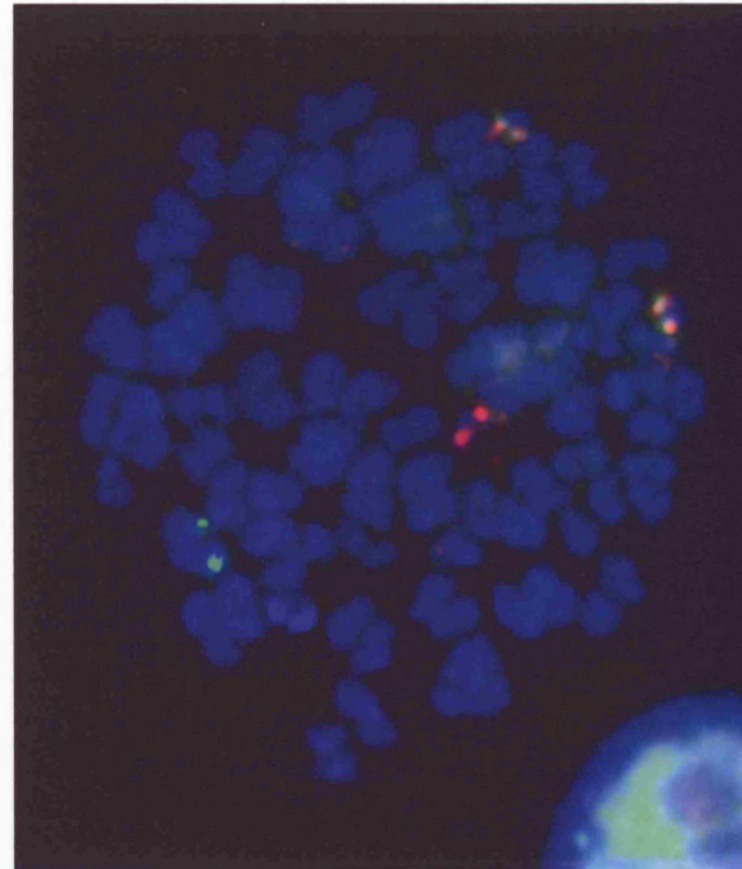
Ho: Hypodiploid (40-45 chromosomes)

Tt: Near Tetraploid (>80 chromosomes)



Finding on interphase

- ● *BCR*
- ● *ABL*
- ● *BCR/ABL fusion*



Findings on metaphase

Fig III:1 *BCR/ABL* positive Hyperdiploid ALL
(case 21 table III:2 UIN 174)

- ● 9 centromere
- ● *BCR*
- ● *ABL*
- ● *BCR/ABL fusion*

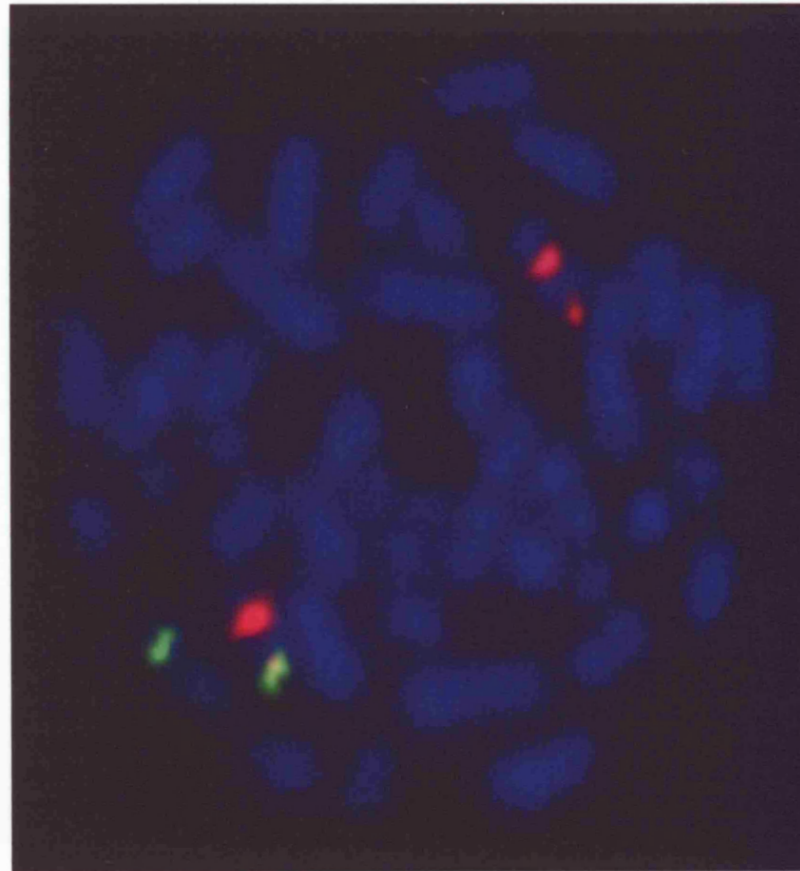


Fig III: 2 *BCR/ABL* positive Ph negative ALL

(case 23 table III:2 UIN 144)

- *AML1* ES
der(12)t(12;21)
- *AML1*
N 21
- *ETV6/AML1 fusion*
der(21)t(12;21)

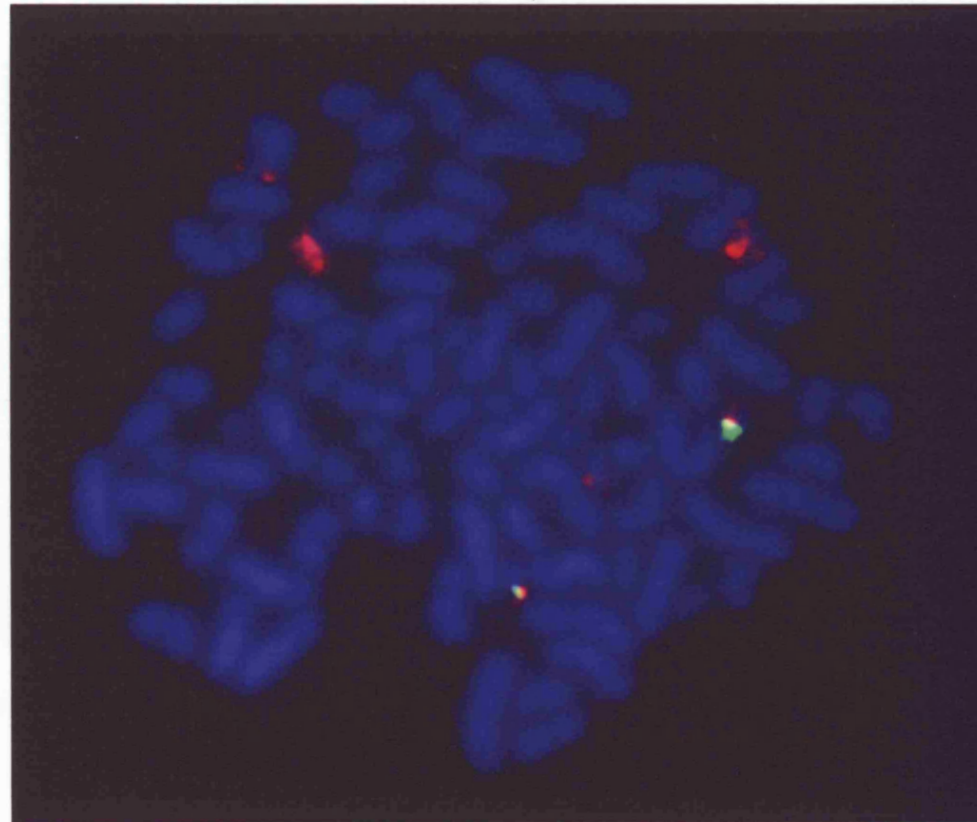
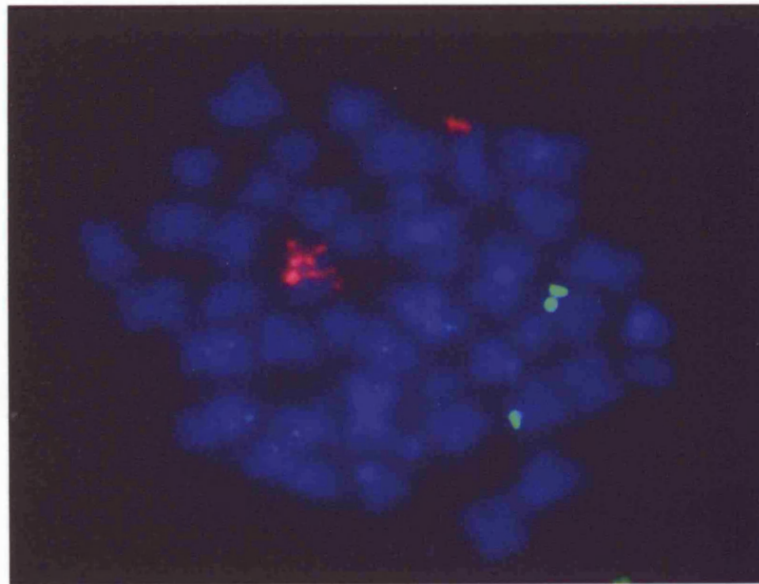


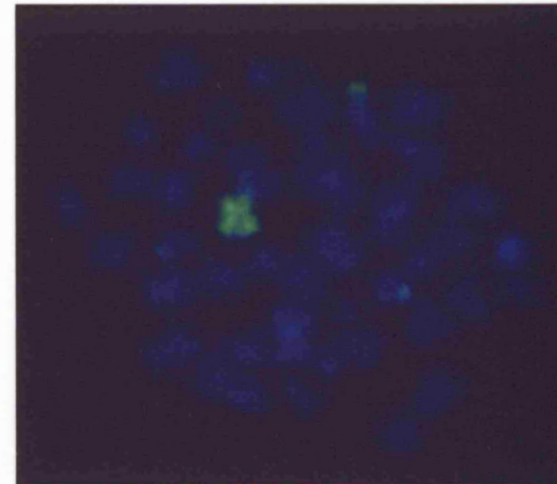
Fig III:3 *ETV6/AML1* fusion positive near tetraploid ALL

(case 33 table III:7 UIN 131)

- *AML1*
N 21
- Amplified
AML1
dup(21)(q)
- *ETV6*
N 12



TEL/AML1 probe



WCP 21

Fig III:4 dup(21)(q) presenting as amplified *AML1*

(case 76 table III:8 UIN:101)

Chapter IV

Interphase FISH in the accurate diagnosis of aneuploidy in adult ALL

IV: 1 Abstract

FISH was used to complement cytogenetic analysis on diagnostic bone marrow or blood from 193 adult patients with ALL. Interphase FISH was carried out to search for aneuploidy in 77 cases with a failed or normal cytogenetic analysis, using a novel device for all chromosome centromeres (Multiprobe®-I system). Complete or partial analysis was possible in 48 cases and revealed abnormalities in 12 (24%) cases. These were high hyperdiploidy (>50 chromosomes) in 5 cases, low hyperdiploidy (47-50 chromosomes) in 4 cases, hypodiploidy (45 chromosomes) in two cases, and near triploidy (>70 chromosomes) in one case. By combining cytogenetic analysis and interphase FISH the overall incidences of the abnormalities in the group thus become hypodiploidy 17/193=9%, low hyperdiploidy 17/193=9%, high hyperdiploidy 18/193=9%, near triploidy 6/193=3%.

The overall incidence of aneuploidy detected in this study was 30%. Of which one third were hidden, detectable only in the non-dividing population.

IV: 2 Introduction

The prognostic significance of chromosomal number for classification of ALL patients was demonstrated in 1978, when an improved prognosis was found in children with ALL and a karyotype of >50 chromosomes (Secker-Walker 1978).

Chromosomal number or ploidy remains an important prognostic feature in ALL.

Loss of chromosomes known as hypodiploidy is generally associated with poor prognosis. It has been reported that the prognosis worsens as the chromosome number declines (Heerema 1999). Near haploidy (23-29 chromosomes) has the worst outcome with median survival of 11 months (Gibbons 1991; Harrison 2004).

A poor outcome is also associated with less than 45 chromosomes in childhood ALL (Heerema 1999), and loss of chromosome 7 in adults (Wetzler 1999). In contrast chromosomal gain, particularly high hyperdiploidy is associated with a better prognosis in both children and adults (Jackson 1990; Secker-Walker 1997). Within the hyperdiploidy groups gains of specific chromosomes are related to the clinical course of disease, the combination of trisomies for chromosomes 4 and 10 predicts good prognosis (Harris 1992), and the presence of trisomies for chromosomes 4 and 18 in high hyperdiploidy has been found to predict five year survival of 96% (Moorman 2003) whereas the presence of trisomy 5 confers poorer outcome within the high hyperdiploidy group in children (Heerema 2000). Low hyperdiploidy (47-50 chromosomes) appears to predict improved outcomes in adults (Secker-Walker 1997).

Unlike chromosomal translocations, associated with the production of chimeric RNA, which can be traced using PCR based methods, detection of numerical

changes cannot be carried out using this molecular technique. Alternative methods, such as the measurement of DNA index or FISH are the most appropriate tests. Measurement of DNA index has limitations, since individual chromosomal gains are not identified, nor is it sensitive enough to detect all cases of low hyperdiploidy, or single chromosome gains or losses. FISH using probes directed to the alpha satellite regions of the individual chromosome centromeres has been used extensively to determine chromosomal gains and losses in interphase cells (Jenkins 1992; Kibbelaar 1993; Tosi 1994; Ritterbach 1998).

By using selected centromeric probes to detect the chromosomes most commonly present within hyperdiploid clones it can be applied at presentation (Ritterbach 1998). The combination of two or three probes has also been described in the detection of minimal residual disease, when a hyperdiploid clone was identified at diagnosis (Heerema 1993; Kasprzyk and Secker-Walker 1997).

In this section of the study interphase FISH using centromeric probes, either as the total chromosome complement together in the Multiprobe-I device or individually / in pairs has been carried out to evaluate this procedure in the detection of chromosomal gains or losses in adult ALL.

IV: 3 Materials and Methods

IV: 3: 1 Patients and controls

Of the 193 patients in this study, in 82 initial cytogenetic analysis failed or showed only normal metaphases. Diagnostic samples were available from 77 of these patients, bone marrow (n=63), peripheral blood (n=9) and both (n=5). These included 69 entered to the current UKALLXII treatment trial and eight treated independently.

Five bone marrow samples were selected as normal controls as described in Chapter II:1, and an additional three from adults with ALL with pseudodiploid karyotypes (structural chromosomal abnormalities but only 46 chromosome). Positive controls were two adults with ALL and hyperdiploidy seen by conventional cytogenetics.

IV: 3: 2 Probes for FISH

Multiprobe®-I device (Cytocell):

This device allows the simultaneous detection of centromeres from 24 individual chromosomes in a single hybridisation on one slide, using directly labelled centromeric probes. (Details of probes used in the kit are presented in Chapter II:4).

Other probes:

Probes for *BCR/ABL*, *MLL* and *TEL/AML1*, and wcp were applied to the same samples used in interphase FISH studies on cases presented in this section as detailed in Chapter II:4).

IV: 3: 3 Methods of Analysis

Details of cytogenetic analysis, immunophenotyping, FISH and equipment used for analysis are presented in Chapter III: 3: 3.

Interphase FISH

Multiprobe®-I device (Cytocell):

One hundred nuclei were scored for each individual centromeric probe. The hybridisation efficiency of the five control samples, ranged from 47-94%. The proportion of nuclei with two signals was also variable ranging from 83-98% as detailed in (Tables II.2 and II.3). Samples were only included in the study if a successful result was achieved on a minimum of 10 probes. Those probes that failed most frequently were those for chromosomes 1, 20, 4, 14/22, X, and 13/21 as detailed in (Table II.4).

IV: 4 Study results

Samples were available from 77 patients. In 29 cases, 10 of which had a normal karyotype and 19 with a failed cytogenetic result, material was inadequate for testing with Multiprobe-I. In the remaining 48 cases, 28 with a normal karyotype, and 20 with a failed cytogenetic result a successful FISH result was obtained.

These were common-ALL (n=19), pre-B ALL (n=7), T-ALL (n=12), Null ALL (n=1), and “other” (n=2). Immunophenotyping was not available for the remaining seven patients.

Aneuploid cases

In 36 cases, two signals were observed for all chromosomes tested with Multiprobe-I, indicating a normal diploid number of chromosomes. In 12 cases the gain or loss of signals implicating the gain or loss of whole chromosomes. The abnormal results from Multiprobe-I are shown in Table VI.1.

In two cases (A-B) there was loss of one centromeric signal for chromosomes 9 and 13/21 respectively (case B had a single copy of *AML1* making monosomy 21 more likely), the absence of metaphases prevented further detailed studies in these cases. In both cases, a number of probes failed to hybridise, thus losses or gains of other chromosomes were difficult to rule out. Thus the verity of these chromosomal losses is in question. In cases C and D, with apparent gain of chromosome 8 only, the successful hybridisation of other probes supported this positive finding as a true result. Two further cases (E-F) showed five signals on analysis of centromere 13/21, the associated gain of an *AML1* signal in both cases supported the conclusion of an

extra copy of chromosome 21. However the presence of further gains could not be ruled out due to poor quality of the results from other wells.

There were two cases with evidence of a high hyperdiploid clone (modal number >50) (G & H) with gains of centromeric signals corresponding to chromosomal gains commonly found in high hyperdiploidy.

In two cases (I & J), there was a combination of trisomies and tetrasomies for chromosomes within abnormal clones, giving modal numbers of 59-61 in case I, and 63-72 in case J.

Cases K & L were unique in that the chromosomal gains were associated with apparent chromosomal losses. In case K interphase FISH showed the presence of t(9;22) in addition to a hyperdiploid clone and an apparent monosomy of chromosome 7, In case L with apparent monosomies for chromosomes 6 and 13 in addition to tetrasomy X, a small number of metaphases were sequentially hybridized with combinations of wcp's to fully characterise the karyotype of these patient as detailed in Chapter V.

IV: 5 Discussion

Cytogenetic findings in adults identify prognostic subgroups, of which some of the most significant structural abnormalities can be detected by PCR. Numerical chromosomal changes on the other hand can only be detected by conventional cytogenetics and cannot be detected by many molecular procedures. Since karyotypic analysis is often difficult in ALL, and chromosome morphology is notoriously poor in hyperdiploid clones, an alternative method to detect numerical changes is needed. Measurement of the DNA index has its limitations. It will only provide information on total DNA content. Details of any specific gains or losses of individual chromosomes remain unknown.

FISH using centromeric probes is a highly reliable and sensitive method that allows enumeration of individual chromosomes. Previous studies comparing interphase FISH using centromere probes and DNA index showed that FISH is the more sensitive technique (Ritterbach 1998). A number of previous studies have utilised a selected number of centromeric probes as surrogate markers for hyperdiploidy (Kasprzyk and Secker-Walker 1997; Ritterbach 1998). The use of selective probes means that abnormality detection is limited to the chromosomes for which the probes are being applied. The combined testing for all chromosomes in one test is thus an attractive alternative allowing detection of hyperdiploid as well as hypodiploid clones, and identifying isolated gains or losses of one or a few chromosomes.

In this study a series of adult ALL patients were studied for numerical chromosomal abnormalities, using Multiprobe-I. This device is designed to allow testing for all

chromosomes in one hybridisation. In using this technique several problems were encountered. For a single hybridisation all probes must be calibrated to the same conditions. When probes are attached to a single slide this is the responsibility of the manufacturers. There were problems with the availability of the kits, as well as cross hybridisation, which made analysis impossible for chromosomes 5/19, and to a lesser degree, 13/21 and 14/22. Smaller probes, in particular for chromosome 20 centromere, frequently failed due to incomplete hybridisation at conditions adequate for the other probes. The combination of these difficulties meant that in every case at least one probe (that for 5/19) gave an inconclusive result and in many cases up to one third of probes gave inadequate results.

However centromeric probes for the chromosomes most commonly involved in hyperdiploidy (6/10/17/18) (Moorman 1996; Raimondi 1996), as well as the chromosomes of specific interest in adults, namely 7 and 8 (Wetzler 1999) were robust and reliable in standard hybridisation / washing processes and produced a high success rate.

In addition to probe factors, success was related to availability and quality of patient material. Testing for all 24 probes requires a total of 50 µl of high cell density sample, which was not available in a high proportion of patients. This applied in particular in those in which the initial cytogenetic analysis had failed. In some cases the quality of cells was poor, therefore only a small proportion of nuclei hybridised efficiently, yielding too few cells for successful analysis.

Despite these technical difficulties, analysis was possible on 48 patients and within this group 12 cases with abnormalities were found. Of particular interest in nine of

these cases, the first indications of a hidden hyperdiploid clone came from the detection of additional signals with specific probes on screening for structural abnormalities. The most common finding was of an extra copy of *AML1*, present in seven cases, five of which showed classical high hyperdiploidy. This observation reflects the known high incidence of trisomy 21 in high hyperdiploidy (Moorman 1996; Raimondi 1996). Extra copies of *BCR*, *ABL*, *ETV6* and *MLL*, indicating trisomies of chromosomes 22, 9, 12 and 11 respectively were also present in some of these cases.

Three cases were not revealed by specific probes. They were cases with isolated trisomy 8, for which no specific probe was applied, and one case with loss of one chromosome 9 centromere signal. This case was of interest in that the initial cytogenetic analysis had shown only normal metaphases. Interphase FISH however confirmed the presence of a small clone of *BCR/ABL* positive cells accounting for 11-15% of nuclei, without loss of the second *ABL* signal. The additional finding of the loss of one chromosome 9 centromere in 17% nuclei suggested the presence of a further abnormality in addition to the Ph translocation. This hypothesis could not be confirmed due to the lack of metaphases showing *BCR/ABL* fusion or a single chromosome 9 centromere signal.

A similar situation was observed in two further cases with apparent monosomies, these were shown to result from unbalanced translocations with loss of centromeric regions rather than whole chromosomes (as detailed in Chapter V). This emphasises that the interpretation of hypodiploidy from the use of centromeric probes must be undertaken with caution, especially in cases with no metaphases to substantiate the

findings.

In conclusion the utilisation of the Multiprobe-I offers an exciting alternative method of testing for numerical chromosomal abnormalities in ALL. Multiprobe-I is very compact and user-friendly, especially when large numbers of samples are being tested. It is much more convenient and quicker than carrying out individual or paired centromeric probe. Although the method has some limitations in the amount of material required and difficulties of complete analysis, a number of cases with hidden abnormalities were detected. Even in cases with very scant material, the Multiprobe-I provided useful information albeit from a limited selection of probes. The highest came from combining this information with results from tests for *BCR / ABL / MLL / ETV6* and *AML1*, which covered the detection of most major numerical abnormalities.

Table IV.1 Abnormal Multiprobe-I results

	UIN	CC	Number of signals for each centromere on Multiprobe-I (% of nuclei showing abnormal copy number)																			
			1	2	3	4	6	7	8	9	10	11	12	13/21	14/22	15	16	17	18	20	X	Y
A	39	N	F	2	2	F	F	2	2	1 (17)	F	F	F	4	F	2	F	2	2	F	1	1
B	19	F	F	2	F	2	2	2	2	2	F	2	3 (36)	F	2	2	2	2	F	1	1	
C	152	F	2	2	2	2	2	2	3 (47)	2	2	2	2	4	4	2	2	2	F	1	1	
D	147	N	2	2	2	2	2	2	3 (20)	2	2	2	2	F	F	2	2	2	2	2	2	NA
E	110	F	F	2	2	F	F	2	F	2	F	F	2	5 (48)	F	2	F	F	2	F	1	1
F	120	F	F	2	2	F	F	F	2	2	2	2	2	5 (15)	F	F	2	F	2	2	1	1
G	159	N	3 (36)	2	2	3 (55)	3 (55)	2	4 (56)	2	3 (51)	2	2	4	5(37)	2	2	3 (39)	4 (42)	2	F	NA
H	85	F	2	2	2	3 (25)	F	F	2	2	3 (60)	2	2	5 (43)	4	F	2	3 (43)	3 (42)	2	2 (50)	2 (50)
I	21	N	3 (24)	2	2	3 (30)	3 (31)	2	3 (39)	3 (40)	3 (40)	2	2	5 (24)	5(38) 6(22)	3(11)	F	3 (38)	3 (32)	4 (40)	2	NA
J	12	F	F	4 (56)	3 (19) 4 (55)	F	3 (15) 4 (53)	3 (18) 4 (53)	3 (22) 4 (46)	3 (56)	3 (56)	F	F	6 (27)	F	3(25) 4(45)	3(22) 4(37)	3 (25) 4 (40)	3 (18) 4 (42)	4 (33)	2 (50)	2 (24)
K	174	F	3 (29)	3 (34)	4 (33)	3 (23)	2	1 (40)	3 (31)	3 (36)	3 (26)	3 (31)	2	5 (20) 6 (19)	4	3(32)	3(27)	3 (24)	3 (26)	F	2 (35)	1
L	171	F	F	2	2	3 (56)	1 (80)	2	3 (51)	2	3 (55)	2	2	5(79)	4	2	2	3 (44)	3 (46)	2	4 (55)	NA
UIN: Unique identification number			CC: Conventional Cytogenetics					N: Normal Karyotype= 20 normal metaphases							F: Fail		NA: Not Applicable					

Chapter V

Karyotype characterisation in ALL by complementary FISH techniques

V: 1 Abstract

A series of FISH techniques were used to complement cytogenetic analysis in the identification of undefined (marker) chromosomes in 29 adult patients with ALL. Interphase FISH results from a range of specific probes, *BCR/ABL*, *ETV6/AML1* and *MLL*, in addition to centromeric probes, was already available. In 15 patients metaphase analysis provided additional information with these probes. By complementary metaphase FISH analysis with wcps hybridised in a sequential manner, the marker chromosomes were accurately identified, as simple (5 cases) or complex. This study indicated that sequential metaphase FISH analysis is a highly informative approach for the accurate characterisation of karyotypes in ALL and provides a useful alternative to other multicolour FISH techniques.

V: 2 Introduction

The identification of numerical and structural chromosomal abnormalities by cytogenetic analysis continues to be the gold standard approach both for diagnosis and assigning likely outcome of patients as well as the recognition of new recurrent chromosomal abnormalities of prognostic significance. It provides the starting point for molecular studies, leading to identification of genes involved in leukaemogenesis. In ALL the poor quality of metaphases often precludes the accurate characterization of complex karyotypes by conventional chromosomal analysis alone. Molecular cytogenetic techniques have greatly improved the accuracy of detection of important cytogenetic changes, as demonstrated in Chapters III and IV and has allowed the identification of a number of cryptic chromosomal abnormalities in ALL (Romana 1994; Clark 2000).

The use of multiplex FISH (M-FISH), and spectral karyotyping (SKY) analysis has facilitated the accurate definition of complex karyotypes in AML (Tosi 1999), CML (Harrison 2000) and ALL (Mathew 2001).

A number of studies in haematologic malignancies such as AML (Grimwade 1998), myelodysplasia (Ohyashiki 1992), and lymphoma (Offit 1991) have shown a correlation between complex karyotypes and poor outcome.

In ALL the effect of complex karyotypes on outcome remains unclear, although there is some evidence that it is similarly poor (Jarosova 2003).

We have demonstrated that it is possible to completely unravel the make up of highly complex karyotypes in patients with ALL by serial hybridisation of probes to the same metaphase in a methodical and painstaking manner. Locus specific, alpha-

satellite and whole chromosome paints are hybridised serially to a single or a small number of metaphases. This method allows the identification all chromosomes within the metaphase in sequence. Digital images are captured at every step and then superimposed to produce a complete picture of the metaphase (Pinson 2000).

Using this sequential metaphase FISH approach in combination with interphase studies, I analysed a series of 29 ALL patients with incompletely defined karyotypes. The combined techniques allowed characterisation of complex karyotypes in fifteen patients, providing insight into the impact of karyotype complexity in ALL.

V: 3 Materials and Methods

V: 3: 1 Patients and controls

Among 193 patients in this study, 111 had an abnormal karyotype by conventional cytogenetic analysis. Fixed cells from diagnostic bone marrow or peripheral blood samples were available from 24 cases with unidentified marker chromosomes and incomplete karyotypes for further FISH studies. An additional three cases with complete cytogenetic analysis and two cases with a failed cytogenetic result but unexpected interphase FISH findings were also analysed.

V: 3: 2 Probes for FISH

Details of all probes used are presented in Chapter II:4:1).

Specific probes: Commercial probes for *BCR/ABL*, *MLL* and *ETV6/AML1* (Vysis, Downers IL, USA) were used in interphase FISH studies.

Centromere specific probes: Multiprobe®-I (Cytocell) or single indirectly labelled alpha satellite probes (Oncor) were used for chromosome enumeration.

Whole chromosome painting probes: Digoxigenin or biotin labelled wcps were used. (StarFISH, Cambio)

V: 3: 3 Methods of Analysis

Details of cytogenetic analysis, FISH preparation and analysis are presented in Chapter II.

FISH:

All probes were hybridized according to manufacturers' instructions. As detailed in Chapter II.

Interphase FISH

All 29 cases had been tested by interphase FISH for *BCR/ABL*, *MLL* and *ETV6/AML1*. The cases with failed or normal cytogenetics were also studied by Multiprobe-I to enumerate chromosomes, as previously described in Chapters II and III.

Metaphase FISH:

Slides, from 27 patients, which had been previously hybridized with dual-colour probes, had metaphases available, for serial hybridization with wcps. Abnormalities detected by conventional cytogenetic analysis, such as apparently deleted chromosomes, or by interphase FISH with specific probes, such as loss of one signal for *BCR*, *ABL*, *ETV6*, *AML1* or *MLL* directed the choice of initial wcp to be used.

Images of metaphase(s) showing the relevant abnormality with the specific probe were captured. In the next step, two or three wcps specific for the chromosomes of interest were applied using a mixture of biotin (green) and Cy3 (red) labeled probes, combined as 50% biotin: 50% Cy3 (yellow). Images of the same metaphase(s) as captured with the specific probes were also captured following hybridization with wcp. The findings from this step were used to direct the next wcp selection, and so on. After each hybridisation step, a new image of the same metaphase(s) was captured. The images were then digitally merged to produce a composite image of the metaphase as it would have appeared had the wcps been all applied

simultaneously. These steps were repeated until either the whole chromosome marker(s) was elucidated or the material became too friable to proceed with further testing. Up to six hybridisations with wcps were carried out.

In two cases in which initial cytogenetic analysis had failed to produce a result, there were very few metaphases. Interphase FISH showed loss of one or more centromeric signal, suggestive of the presence of hidden monosomies. Wcp for the apparently lost chromosome(s) was applied to a slide with only interphase nuclei. Greater than the expected two distinct areas of signal seen on interphase nuclei, suggested an underlying translocation. Wcp was applied to the slide with the metaphase(s) to confirm the presence of derived chromosomes.

Multicolour-FISH (M-FISH/24-colour FISH):

Another investigator in our laboratory (R.Jalali) carried out M-FISH in four cases, using the 24-colour probe kit (SpectraVysion™, Vysis, U.K.). This allows simultaneous detections of all 24 chromosomes in a single hybridisation and allowed direct comparison with sequential FISH.

V: 4 Study Results

Of the 29 cases selected for further studies, sufficient material was available for further studies on only 15 of them. The details of these cases are presented in (Table V.1).

V: 4: 1 Simple marker chromosomes:

In five cases the unidentified marker chromosome was derived entirely from one chromosome (cases 1-5)

Case 1:

Karyotype by CA: 46 XY,del(7)(p1?5),t(8;22)(q1?1;q13), **-21,+mar**

Karyotype after FISH: 46,XY,del(7)(p1?5),t(8;22)(q1?1;q13),**dup(21)(q?)**

FISH with locus specific probes in case 1 showed four copies of *AML1* in sequence along the marker chromosome. This indicated that wcp 21 was the choice of wcp to apply, FISH with this paint showed the marker chromosome to be composed solely of chromosome 21 material (Fig III: 4).

Case 2:

Karyotype by CA: 46 XY,-17,+**mar**/47,XY,-17,+**mar1**,+**mar2**

Karyotype after FISH: 46 XY,**r(17)**/47,XY,**r(17)**,+**r(17)**

Cytogenetic analysis in case 2 showed two cell populations with loss of one chromosome 17, and one or two unidentified ring chromosomes. FISH with centromeric probe for chromosome 17 showed two populations of interphase cells. In the majority of nuclei, one normal and two smaller signals were seen, and in a smaller proportion of nuclei one normal and one smaller signal were seen, supporting the presence of one or two derived chromosome 17 (Fig V: 1).

Metaphase FISH confirmed the ringed chromosome as the site of the smaller signals.

Case 3:

Karyotype by CA: 45,XY,-7,t(9;22)(q34;q11),-10,+mar

Karyotype after FISH: 45,XY,-7, t(9;22)(q34;q11),del(10)(q?)

In case 3 the karyotype showed a t(9;22)(q34;q11), apparent monosomy of chromosomes 7 and 10, in addition to a marker chromosome. Interphase FISH showed *BCR/ABL* fusion. Metaphase FISH painting with wcp7 and wcp10 confirmed the monosomy 7 and revealed the marker chromosome to be del(10)(q?) (Fig V: 2).

Case 4:

Karyotype by CA: 46,XY,t(2;9;22)(p1?2;q34;q11),-5,del(11)(q14),+mar

Karyotype after FISH: 46,XY,t(2;9;22)(p1?2;q34;q11),del(5)(q?),
del(11)(q21q23)

Cytogenetic analysis in the fourth case showed a complex Ph chromosome, a del(11q), monosomy of chromosome 5, and a marker chromosome. Interphase FISH confirmed the *BCR/ABL* fusion, and two normal *MLL* signals. Sequential metaphase FISH was carried out in a stepwise manner wcp 22 was applied first, followed by wcp 9 and wcp 2 together. The third hybridisation was wcp 5, applied to the same metaphase. This confirmed t(2;9;22) and identified the marker chromosome as a del(5)(q?) (Fig V: 3).

Case 5:

Karyotype by CA:

47,XY,**add(8)(p1?)**,?add(9)(p?),del(9)(p?),**add(12)(p?)**,+21/46,idem,-Y

Karyotype after FISH:

47,XY,**del(8)(p?1)**,?add(9)(p?),del(9)(p?),**t(12;21)(p13;q22)**,+21/ 46,idem,-Y

Cytogenetic analysis in case 5 showed abnormalities of chromosomes 8, 9 and 12 plus an extra copy of chromosome 21. Interphase FISH showed *ETV6/AML1* fusion. Metaphase FISH with wcp8, wcp12 and wcp21 confirmed both t(12;21) and +21 in addition to del(8q)(Fig V: 4).

V: 4: 2 Complex marker chromosomes:

In nine cases (cases 6-15) the unidentified chromosome was shown to be the product of translocations involving between two to five chromosomes.

Case 6:

Karyotype by CA: 46,XX,**add(2)(q3?7)**,-8,**add(9)(p2)**,+mar

Karyotype after FISH: 46,XX,**der(2)t(2;8)(q?;?)**,**del(8)(q)**,
der(9)del(9)(p?)t(2;9)(?;?)

In case 6 cytogenetic analysis showed abnormalities of chromosomes 2 and 9, monosomy for chromosome 8 and a marker chromosome. FISH with wcp 2 and wcp 8, followed by wcp 9 showed three derived chromosomes; a der(2)t(2;8), del(8)(q) and der(9)del(9)(p?)t(2;9) (Fig V: 5).

Case 7:

Karyotype by CA: 46,XY,**der(7)(q21)**/46,XY,**der(7)(q21)**,**del(19)(p11p13)**

Karyotype after FISH: 46,XY,**der(7)t(4;7)(p?;q?)/46,XY,der(7)t(4;7)(p?;q?),**
t(11;19)(q23;p?)

Cytogenetics in case 7 revealed abnormalities of chromosomes 7 and 19. Interphase FISH showed three signals with single-colour *MLL* probe. Metaphase FISH showed splitting of the probe between two chromosomes confirming a rearrangement of *MLL*. wcp11 with wcp19, wcp 4 with wcp7 were used in sequence and revealed the *MLL* rearrangement to result from t(11;19) with der(7)t(4;7) (Fig V: 6).

Case 8:

Karyotype by CA: 47,XY,**del(3)(q22),del(6)(q13),-8,t(11;14)(q13;q32),**
+mar1,+mar2

Karyotype after FISH: 47,XY,**+3,del(3),del(6),der(8)t(8;3;8;19),t(11;14)(q13;q32)**

Cytogenetic analysis in case 8 showed monosomy 8, deletions of chromosomes 3 and 6, and two marker chromosomes. FISH with wcp3, wcp6 and wcp8, followed by alpha satellite probe to chromosome 8 centromere in one metaphase, confirmed the del(3) and del(6), and showed the mar1 to be an extra copy of chromosome 3, the mar2 was a derived chromosome 8 with insertion of chromosome 3 material (Fig V: 7a, 7b). Further testing with wcp22, followed by wcp1 and wcp19, identified the second marker chromosome as a der(8)t(8;3;8;19) (Fig V: 7c).

Case 9:

Karyotype by CA: 46,XX,**add(2)(p?),add(3)(p?)-5,-6,add(7)(q?),i(9)(q10),**
-13,add(14),der(14;17),+mar1,+mar2,+mar3,+mar4

Karyotype after FISH: 46,XX,**add(2)(p?),del(3)(p?),der(5)ins(5;3)(?;??),**
der(5)t(?;5)(?;?),del(6q),add(7)(?q),i(9)(q10),
der(13)t(2;13)(?;?),der(17)t(14;17)(?;?)

The karyotype in case 9 was highly complex, with apparent losses of chromosomes 5, 6, and 13, abnormalities of chromosomes 2, 3, 7, 9, 14 and 17 in addition to four marker chromosomes. Interphase FISH showed three copies of *ABL* consistent with the cytogenetically identified i(9)(q10). Metaphase FISH with wcp3, wcp5 and wcp14, followed by wcp7 and wcp17 was undertaken. Results from wcp7 were inconclusive, however the other tests confirmed add(2) and add(3), and showed four derived chromosomes; der(2)t(2;13), der(5)ins(3;5), der(5)t(?;5), der(?)t(7;?), del(6) and confirmed der(17)t(17;14) (Fig V: 8). Results from M-FISH of this case were concordant in part and parallel examination of metaphases from both techniques provided more detailed information. The der(5) was identified as der(5)ins(5;3), the der(17) identified as der(17)t(17;14), as well as defining a del(5), a der(7)t(7;22), a der(14)t(14;6), and a der(14)t(14;5).

By M-FISH a small marker was described as der(2)t(2;22). A marker of similar size appeared to be a der(2)t(2;13) by sequential FISH.

Case 10:

Karyotype by CA: 46,XX,der(5)t(1;5)(q23;q35)/45,XX,-2,**add(4)(p?),-5,-8,-11,**
add(12)(p1),del(12)(p1p1),+mar1,+mar2,+mar3,+mar4

Karyotype after FISH 46,XX,der(5)t(1;5)(q23;q35)/ 45,XX,der(5)t(1;5),
der(2)t(2;5)(?;?),der(2)t(12;?;5;2)(?;?;?;?),
der(4)ins(4;5)(?;??),-5,der(8)t(8;12),-12,der(12)t(5;12)

Cytogenetic analysis of case 10 showed a complex karyotype with two populations of abnormal cells, one with an isolated der(5)t(1;5), the second showing losses of chromosomes 2, 5, 8, possibly 11, abnormalities of chromosomes 4 and 12 with four marker chromosomes. Interphase FISH showed two normal copies of *MLL*. One slide was hybridised in five sequential steps, with wcp1 and wcp5, followed by wcp2 and wcp8, then wcp4 and wcp12, followed by wcp11 and wcp17 and finally wcp19. All but the last experiment were successful, and two abnormal clones were identified, one with isolated der(5)t(1;5) (Fig V: 9a). The second clone with five derived chromosomes der(2)t(2;5), der(?2)t(12;?5;2), der(4)ins(4;5), der(8)t(8;12), and der(12)t(5;12) (Fig V: 9b), M-FISH of this case confirmed the findings in the first clone but no metaphases showing the complex clone were observed.

Case 11:

Karyotype by CA: 45,X,X,add(3)(q12),del(6)(q?16),add(9)(q?),
add(12)(p11)/45,idem,del(6)(q?16),
der(6)del(6)(q?16)rdup(6)(pter-q12)

Karyotype after FISH: 45,X,-X,der(3)t(3;21),r(6),der(9)t(9;3),
der(12,)t(9;3;21;12), der(12)t(12;21)

Cytogenetics showed abnormalities of chromosomes 3, 6, 9 and 12. Interphase FISH detected an *ETV6/AML1* fusion signal in all nuclei, which on metaphase FISH analysis appeared to be localized on the marker chromosome. The karyotype established by M-FISH was: 46,XX,der(3)t(3;21),r(6),der(9)t(9;3),
der(12)t(9;3;21;12),der(12)t(12;21) (FigV: 10a).

Sequential FISH with wcp2 and 12, showed the q arm of the marker chromosome to

contain material from both chromosomes (Fig V: 10b), Attempts to re-hybridise this metaphase to wcp3 and 6 failed.

Case 12:

Karyotype by CA: 46,XX,ins(1;?)(q21;?),del(6)(q14)/47,idem,+mar

Karyotype after FISH: 46,XX,ins(1;?)(q21;?),del(6)(q14)/
47,add(1),del(6)(q),der(?)ins(?;6)

Sequential FISH was incomplete; abnormalities of chromosomes 2 and 6 in association with a marker chromosome were identified by cytogenetic analysis. Metaphase FISH with wcp1 and wcp6 showed add(1) and confirmed del(6). The marker chromosome appeared to have an insertion of material from chromosome 6 into the q arm; further hybridization to identify the origins of this marker was unsuccessful (Fig V: 11).

Case 13:

Karyotype by CA: 46,XY,del(13)(q12q14)

Karyotype after FISH: 46,XY,der(9)?ins(9;22)(q34;q11q11),del(13)(q?),
ins(14;13)(q?;q?),del(22)(q?)

The only abnormality detected by cytogenetic analysis was a deleted chromosome 13. Interphase FISH showed a *BCR/ABL* fusion, and metaphase FISH with *BCR/ABL* locus specific probe and chromosome 9 alpha satellite centromeric probe showed the fusion signal to be located on the long arm of chromosome 9 (Fig III: 2). Additional testing with wcp13 and 14 revealed a der(14)ins(14;13) in addition to the del(13)(q) (Fig V: 12).

M-FISH confirmed these abnormalities.

Case 14:

Karyotype by CA: Fail

Karyotype after FISH: **45,XY,der(3)t(3;7),-7,t(9;22)(q34;q11)/64,idem,+X,
+1,+2,+3,der(3)t(3;7),+der(3)t(3;7),+4,+5,+7,+8,+9,
+10,+11,+15,+16,+17,+18,+19,+21,+der(22)t(9;22)**

This case failed cytogenetic analysis. Hyperdiploidy was detected by interphase FISH. The chromosomal gains were associated with apparent monosomy 7. Wcps showed the presence of four signals for chromosome 7 in interphase nuclei and one metaphase. Sequential FISH on three metaphases with 3-6 hybridisation rounds per metaphase was used to further define the karyotype. Due to the paucity of metaphases, the initial step in the identification of partner chromosomes was to use wcp applied to interphase nuclei (as described in section V:3:3). The metaphases were analysed using *BCR/ABL* probe and whole chromosome paint for chromosomes 1, 3, 4, 5, 7, 8, 9, 11, 14 and 22. By combining the interphase FISH results with those of the metaphase wcp the karyotype was defined as:

**45,XY,der(3)t(3;7),-7,t(9;22)(q34;q11)/64,idem,+X,+1,+2,+3,der(3)t(3;7),
+der(3)t(3;7),+4,+5,+7,+8,+9,+10,+11,+15,+16,+17,+18,+19,+21,+der(22)t(9;22).**

(Fig V: 13)

Case 15:

Karyotype by CA: Fail

Karyotype after FISH: **54,+der(X)(X;13)x2,+4,+6,+der(?)t(?;6),+8,+10,
+der(13)t(6;13),+17,+18,+der(?)t(?;6)(?;?)**

A hyperdiploid clone with gains of chromosomes X, 4, 8, 10, 17, 18, 21 and

apparent monosomies for 6 and 13 were detected by interphase FISH.

Sequential FISH experiments on two metaphases and a number of interphase cells using whole chromosome paints for chromosomes X, 1, 4, 6, 9, 11 and 13 was carried out (Fig V: 14a) and revealed two der(X)t(X;13) chromosomes, der(13)t(6;13), and one derived chromosome containing chromosome 6 material defining the karyotype as: 54,+der(X)(X;13)x2,+4,+6,+der(?)t(?;6),+8,+10,+der(13)t(6;13),+17,+18 (Fig V: 14b).

V: 5 Discussion

A combination of interphase and metaphase FISH in a serial manner was carried out in 15 cases with incompletely characterised karyotypes, in an attempt to fully identify marker or unidentified chromosomes. The group was heterogeneous in terms of karyotype complexity. Despite the fact that analysis was dictated by the availability of material for further studies, a number of common features were found. Sequential FISH revealed that in a high proportion of the cases with suspected partial or total loss of a chromosome, from cytogenetic analysis or FISH with centromeric probes, hidden and occasionally complex translocation were found. This confirms the known higher incidence of structural rather than numerical chromosomal changes in ALL. A similar observation has been described in patients with dic(9;20) who present as monosomy 20 (Clark 2000). This observation is important when interpreting apparent monosomy detected by interphase FISH, in the absence of metaphase analysis.

These studies also showed a pattern of involvement of certain chromosomes in these complex translocations. Chromosomes 3, 5, 8 and 12 were involved in three cases each, and chromosomes 2, 7, 9, 13, 21 and 17 involved in two cases each. Abnormalities involving chromosomes 3 and 5 have not been commonly described in ALL (Chen 1992; Mitelman 1995; Mitelman 2004). Chromosome 5 abnormalities reported in ALL were usually in the form of deletions of the long or short arm and inversion of the p arm del(5p), del(5q), and inv(5p). Monosomy 5 and del(5q) have been associated with a poor outcome in AML (Grimwade 1998). A translocation t(1;5)(q23;q33) has been previously reported in three cases of ALL, all three patients

showed chemotherapy resistant disease and short survival, (Barriga 1996).

Abnormalities of chromosome 8 are often seen in AML (Grimwade 1998). In ALL trisomy 8 has been associated with poor outcome (Wetzler 1999).

Chromosome 12 abnormalities in ALL frequently involve 12p11-13 in balanced translocations and deletions (Mitelman 1995; Mitelman 2004). The well known t(12;21)(p13;q22) in childhood ALL and dic(9;12)(p13:p11), including other abnormalities of (12p), are associated with a more favourable outcome in ALL (Behrendt 1995; Harbott 1997; Secker-Walker 1997).

The effect of highly complex karyotypic abnormalities on outcome in ALL remains unclear and is likely to depend on the primary chromosomal changes. In this small series, five of the 15 cases had evidence of hidden abnormalities, associated with a poor prognosis, four had *BCR/ABL* fusion and one had an *MLL* rearrangement.

However half the patients with complex chromosomal abnormalities without *BCR/ABL* fusion or *MLL* rearrangement had disease relapse within one year of presentation.

The availability of revolutionary new multicolour FISH techniques including M-FISH, makes it possible to investigate all 24 chromosomes (22 autosomes, X and Y) in a single hybridisation. However the success of M-FISH is dependent on the number and quality of abnormal metaphases. The complexity of the probe mixture, including 24 chromosome paints, puts enormous dependence on computer software to identify the origin of chromosomal segments. Well spread chromosomes with minimum overlapping is essential to prevent interaction between pseudocolours. In our small series, four cases were tested by both M-FISH and sequential FISH. The

findings were identical in one case with abnormalities of only three chromosomes. In two cases with more than four chromosomes involved in complex translocations, there was less correlation between the two approaches. The sequential approach was more accurate as it was certain which chromosome wcp had been applied. M-FISH showed interference between signals in poor quality metaphases in these cases.

Each technique has its limitations. In cases with adequate metaphases, M-FISH allowed complete clarification of karyotypes with more than three chromosomes involved in abnormalities, while sequential FISH became technically more difficult with successive hybridisations. However, the application of sequential FISH provided greater information on a larger number of translocations and identified derived chromosomes with greater accuracy.

In five cases with simpler karyotypes and two cases with failed cytogenetics due to very poor quality metaphases, sequential FISH provided detailed information from a small number of metaphases, which had already been utilized in interphase screening tests. M-FISH was not applicable to these cases.

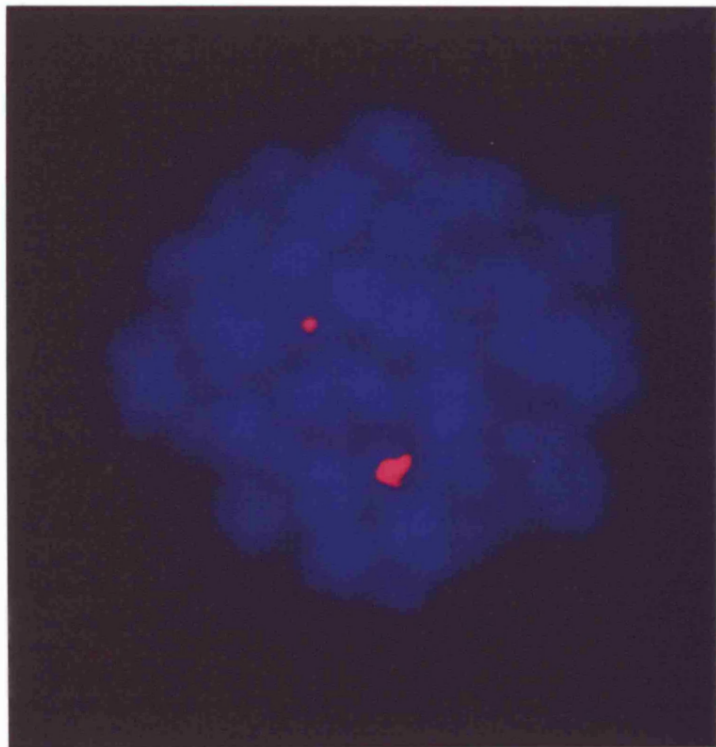
In ALL where metaphase quality and quantity may preclude adequate M-FISH analysis, we have shown that sequential FISH with locus specific, alpha satellite and whole chromosome paints applied to interphase cells and to limited numbers of poor quality metaphases, can be highly informative in the characterization of complex karyotypes.

Further analysis of karyotypes using this highly sensitive sequential interphase and metaphase FISH approach will assist in determining the significance of karyotype complexity in ALL.

Table V.1 Marker chromosomes identified by FISH

			Interphase FISH			Centromere	Metaphase FISH	NCI	
			<i>BCR/ABL</i> F N	<i>MLL</i>	<i>ETV6/AML1</i> F N				
1	101	46,XY,del(7)(p1?5), t(8;22)(q1?1;q13),-21,+mar	N	2	N		46,XY,del(7)(p1?5),t(8;22)(q1?1;q13), dup(21)(q?)	4	EFS 22+
2	56	46,XY,-17,+mar[3]/47,XY,-17, +mar1+mar2[5]/46,XY[3]	N	2	N	D17Zx4	46 XY,r(17) / 47,XY,r(17),+r(17)	1	No CR
3	88	45,XY,-7,t(9;22)(q34;q11), -10,+mar[9]	P	2	N		45,XY,-7,t(9;22)(q34;q11), del(10)(q?)	4	BMR 15
4	91	46,XY,t(2;9;22)(p1?2;q34;q11), -5,del(11q14),+mar[15]/46,XY[3]	P	2	N		46,XY,t(2;9;22)(p1?2;q34;q11), del(5)(q?),del(11)(q21q23)	5	DICR 11
5	82	47,XY,add(8)(p1?),?add(9)(p?), del(9)(p?),add(12)(p?),+21[13]/ 46.idem,-Y[11]/46,XY[3]	N	2	P		47,XY, del(8)(p1?) ,?add(9)(p?), del(9)(p?),t(12;21)(p13;q22),+21/ 46.idem,-Y	4-5	EFS 27+
6	107	46,XX,add(2)(q3?7),-8, add(9)(p2),+mar[20]	N	2	N		46,XX, der(2)t(2;8)(q ?;?) , del(8)(q?) , der(9)del(9)(p?)t(2;9)(q?;?)	3	EFS 20+
7	169	46,XY,der(7)(q21)[3]/ 46.idem,del(19)(p11p13)[15] 46,XY[2]	N	3	N		46,XY, der(7)t(4;7)(p?;q?) / 46,XY, der(7)t(4;7)(p?;q?) , t(11;19)(q23;p13)	4	Uk
8	86	47,XY,del(3)(q22),del(6)(q13),-8, t(11;18)(q13;q23),+mar1,+mar2	N	2	N	D8Z1x2	47,XY,+3, del(3)(q22),del(6)(q13) , der(8)t(8;3;8;19)(?;?;?;?) , t(11;14)(q13;q32)	6	EFS 26+
9	164	46,XX,add(2)(p?),add(3)(p?), -5,-6,add(7)(q?),i(9)(q10),-13, add(14),der(14;17), +mar1,+mar2,+mar3,+mar4	N	3 (50%)	2	N	46,XX,add(2)(p?),del(3)(p?), der(5)ins(5;3)(?;??),der(5)t(?;5)(?;?) ,del(6)(q?),add(7)(?q), i(9)(q10), der(13)t(2;13)(?;?) , der(17)t(14;17)(?;?)	8	Uk

10	33	46,XX,der(5)t(1;5)(q23;q35[7])/45,XX,-2,add(4)(p?),-5,-8,-11,add(12)(p1),del(12)(p1p1),+mar1,+mar2,+mar3,+mar4[4]/46,XX[8]	N	2	N			46,XX,der(5)t(1;5)(q23;q35)/45,XX,der(5)t(1;5),der(2)t(2;5)(?;?),der(2)t(12;?;5;2)(?;?;?;?),der(4)ins(4;5)(?;?;?),-5,der(8)t(8;12),+12,der(12)t(5;12)(?;?)	6	BMR 11
11	100	45,X,-X,add(3)(q12),del(6)(q?16),add(9)(q),add(12)(p11)[18]/45,idem,del(6)(q?16),der(6)del(6)(q?16)rdup(6)(pter-q12)[2]	N	2	P			45,X,-X,der(3)t(3;21)(q1?q22),r(6),der(9)t(9;3)(q2?q1?),der(12)t(9;3;21;12)(q1 ?;q2 ?p13),t(12;21)(p13;q22)	5	BMR 11
12	119	46,XX,ins(1;?)(q21;?),del(6)(q14)[3]/47,idem+mar[4]	N	2	N			46,XX,ins(1;?)q21;??),del(6)(q14)47,idem,der(?)ins(?;6)(?;??)	3	BMR 7
13	144	46,XY,del(13)(q12q14)[20]	P	2	N			46,XY,der(9)ins(9;22)(q34;q11q11),ins(14;13)(q?;q?)	4	BMR 6
14	171	Fail	P	3	N	3(11%) 4(32%)	HeH Multiprobe-I	45,XY,der(3)t(3;7)(?;?;?)-7,t(9;22)(q34;q11)/64,idem,+X,+1,+2,+3,der(3)t(3;7),+der(3)t(3;7),+4,+5,+7,+8,+9,+10,+11,+15,+16,+17,+18,+19,+21,+der(22)t(9;22)	4	Uk
15	174	Fail	N	2	2	N	4(72%) HeH Multiprobe-I	54,+der(X)(X;13)(?;?)x2,+4,+6,+8,+10,+der(13)t(6;13),+17,+18,+der(?)t(?;6)(?;?)	4	Uk
F: fusion signal		ABL: copy no of ABL		MLL: copy no of MLL		AML1: copy no of AML1				
NCI: No of chromosomes involved		HeH: high hyperdiploidy (51-65 chromosomes)				EFS: event free survival (presentation to relapse/death/last visit)				
CR: complete remission		BMR: bone marrow relapse		DICR: died in CR		Uk: unknown				



17 centromere probe

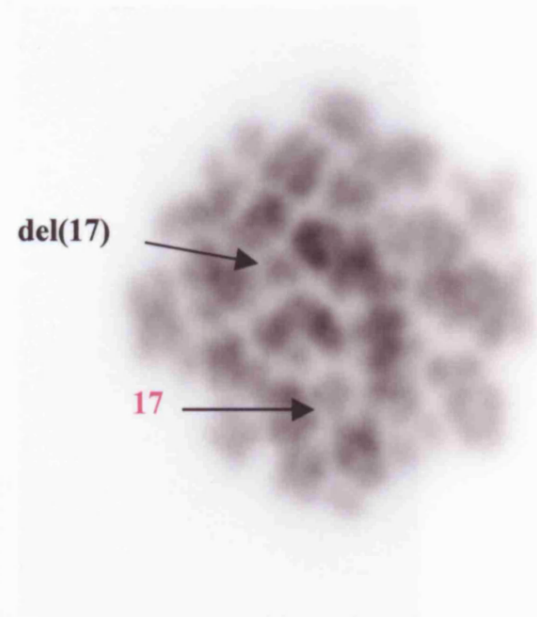
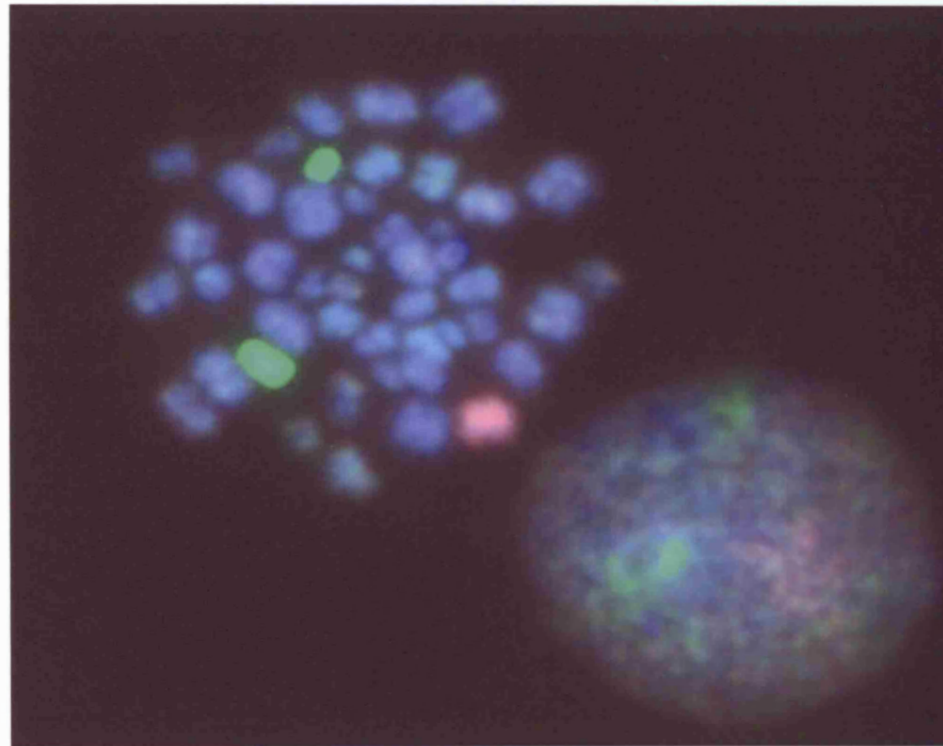


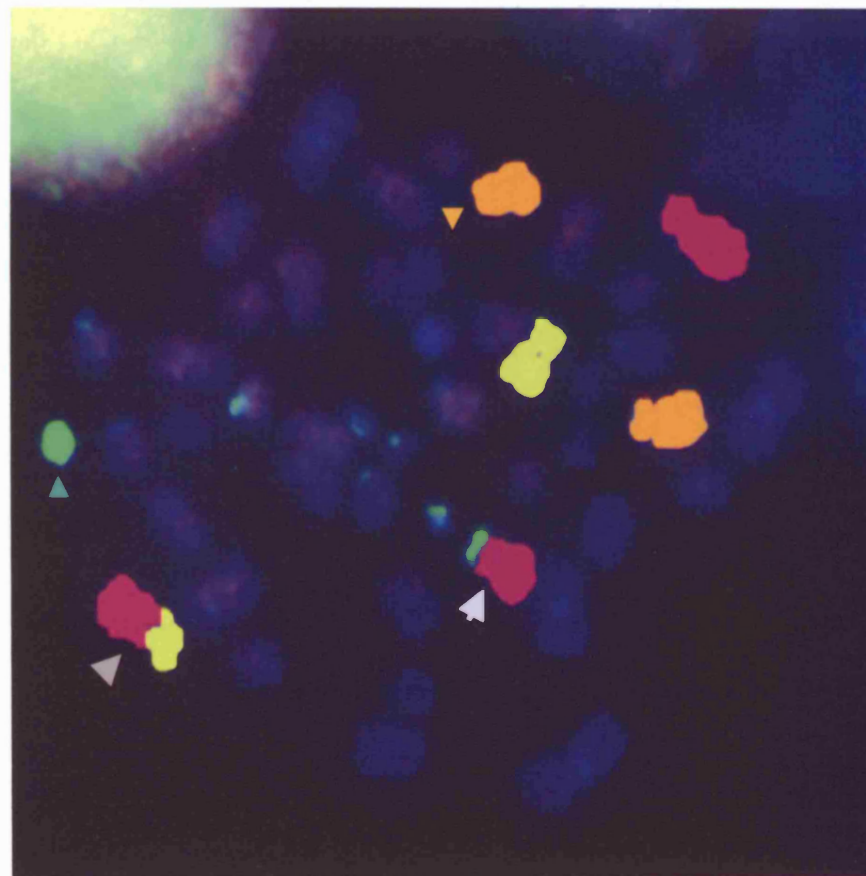
Fig V:1 derived chromosome 17 marker
(case 1 table V:1 UIN:56)



WCP 7 WCP 10

Fig V:2 Monosomy 7, and del(10)(q) in Ph positive ALL
(case 3 table V.I UIN:88)

- ▲ $\text{del}(5)(q)$
- ▲ $\text{der}(2)t(2;22)$
- ▲ $\text{der}(9)t(2;9)$
- ▲ Ph

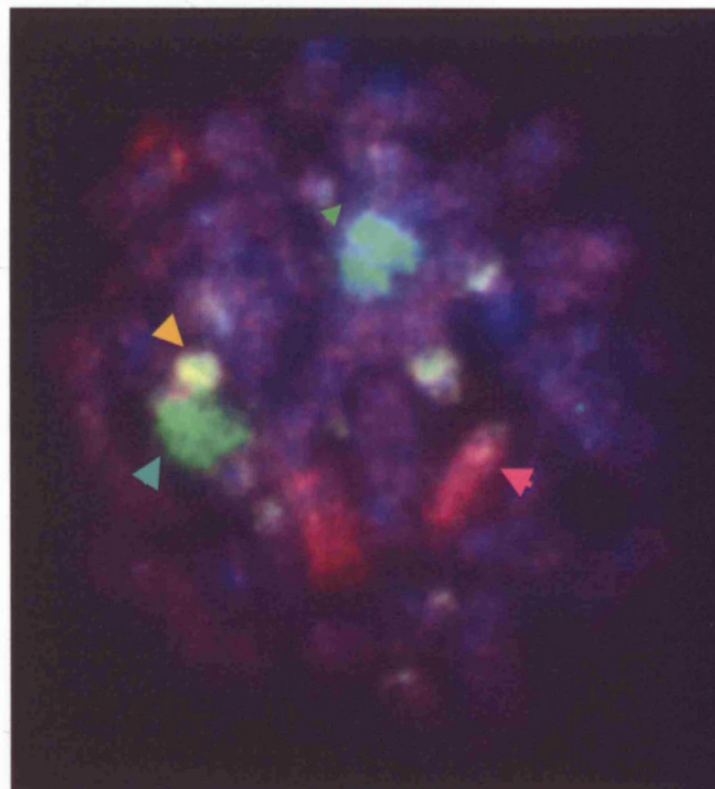


WCP 2 WCP 5 WCP 9 WCP 22

Fig V:3 Variant Philadelphia chromosome with $\text{del}(5)(q)$

(case 4 table V:1 UIN:91)

- ↑ **del(8)**
- ▲ **der(12)t(12;21)**
- ▲ **der(21)t(12:21)**
- ▲ **Normal chromosome 8**

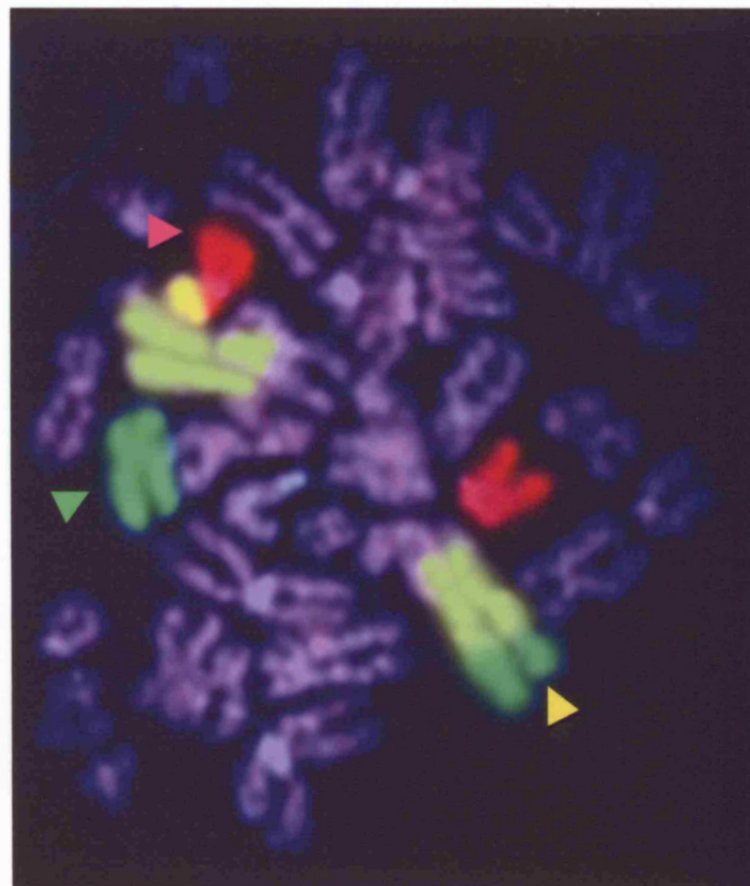


WCP 8 WCP 12 WCP 21

Fig V:4 t(12;21) and del(8)

(case 5 table V:1 UIN:82)

- ▲ **der(2)t(2;8)**
- ▲ **del(8)**
- ▲ **der(9)t(2;9)**

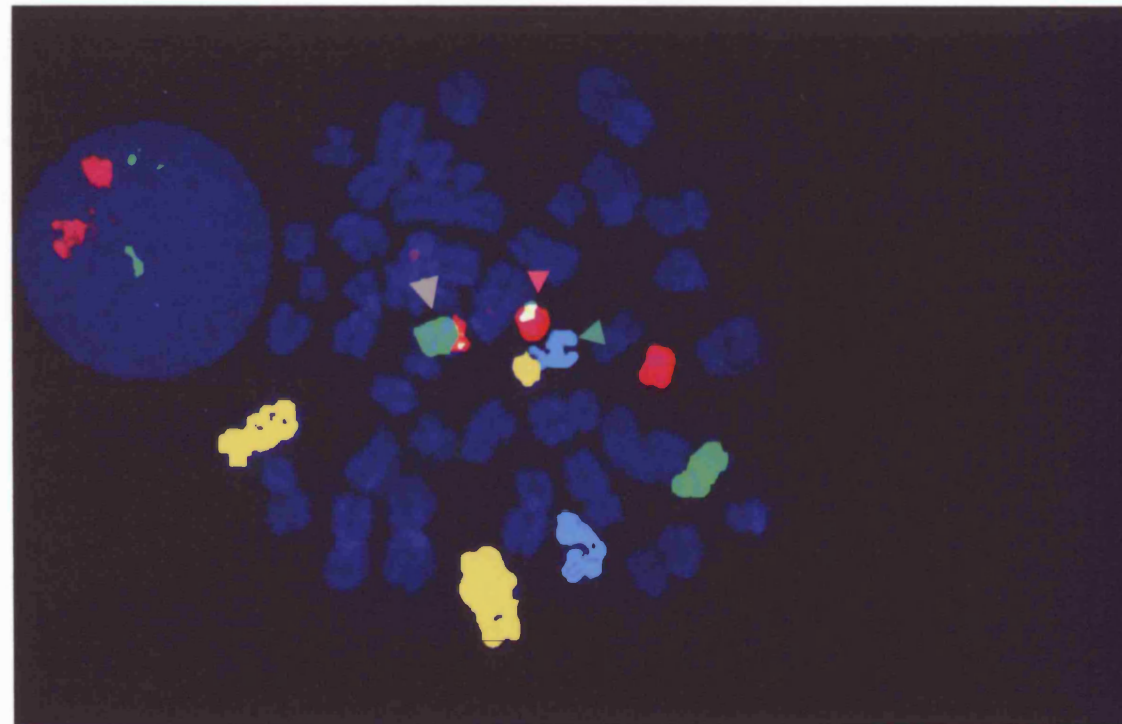


WCP 2 WCP 8 WCP 9

Fig V:5 Three markers

(case 7 table V:1 UIN:107)

- ▲ der(7)t(4;7)
- ▲ der(11)t(11;19)
- ▲ der(19)t(11;19)



WCP 4 WCP7 WCP 11 WCP 19

Fig V:6 Hidden t(11;19) and der(4)t(4;7)
(case 7 table V:1 UIN 169)

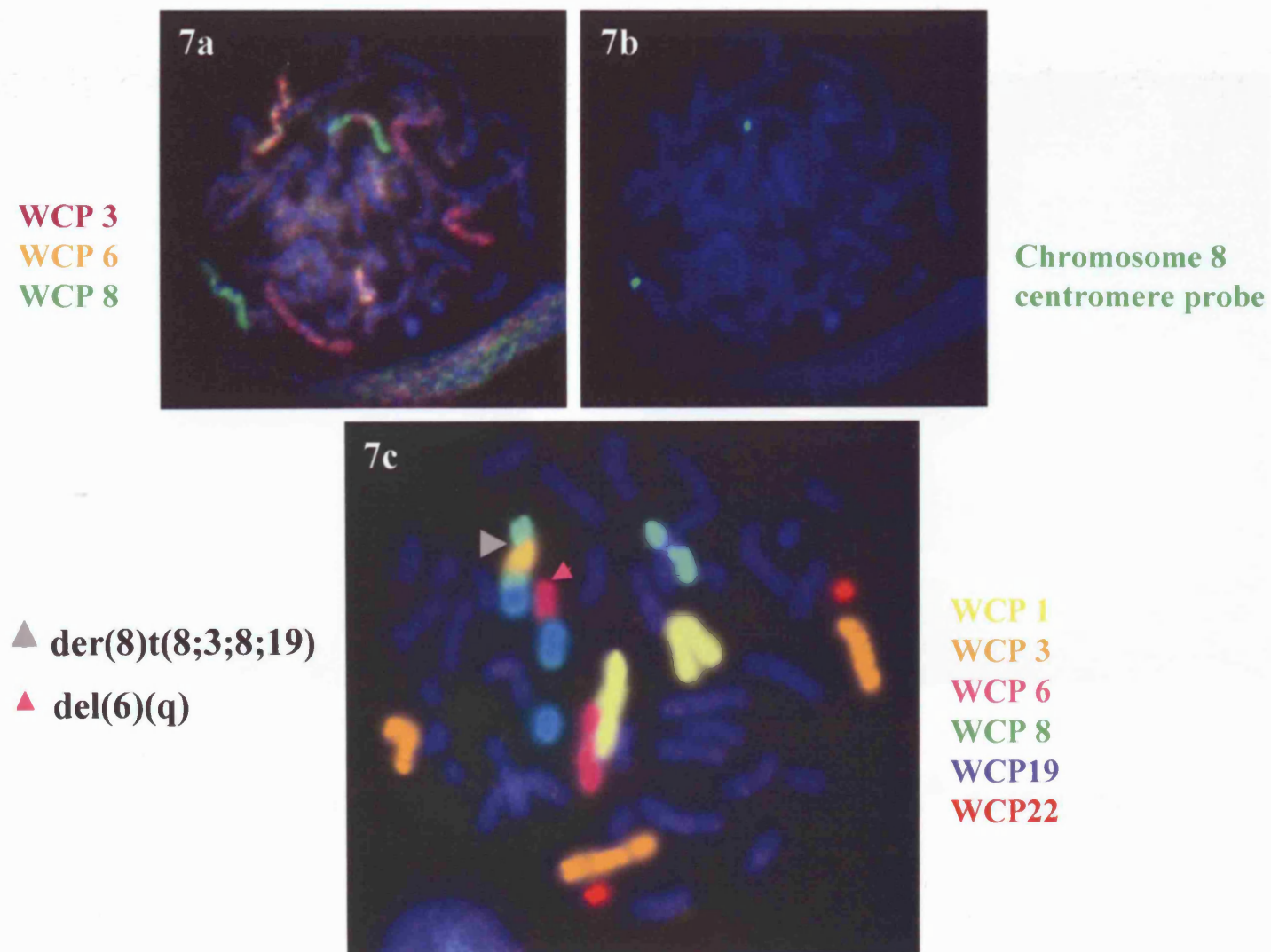
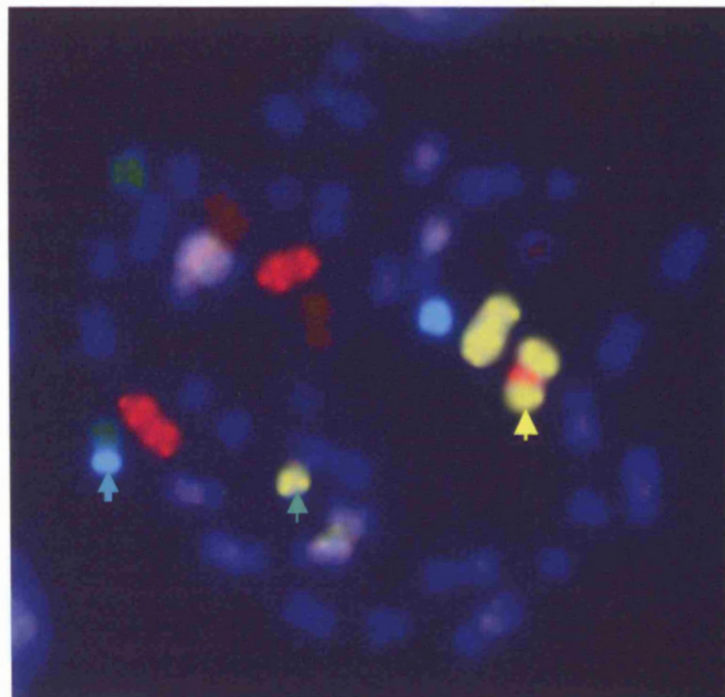


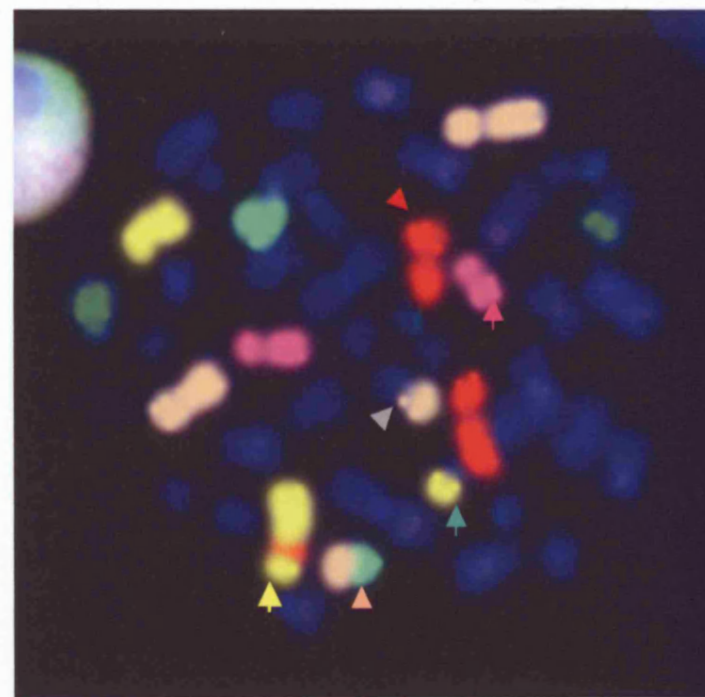
Fig V:7 Complex marker derived from chromosomes 3, 8 and 19

(case 8 table V:1 UIN:86)



WCP 3 WCP 5 WCP 7 WCP 14 WCP 17

▲ der(2)t(2;13) ▲ add(2)(p?) ▲ del(3)(p?) ▲ der(5)ins(5;3) ▲ der(5)t(?;5)
 ▲ del(6)(q?) ▲ der(17)t(14;17)



WCP 2 WCP 3 WCP 5 WCP 6 WCP 13 WCP 14

Fig V:8 Markers by sequential FISH

(case 9 table V:1 UIN:164)

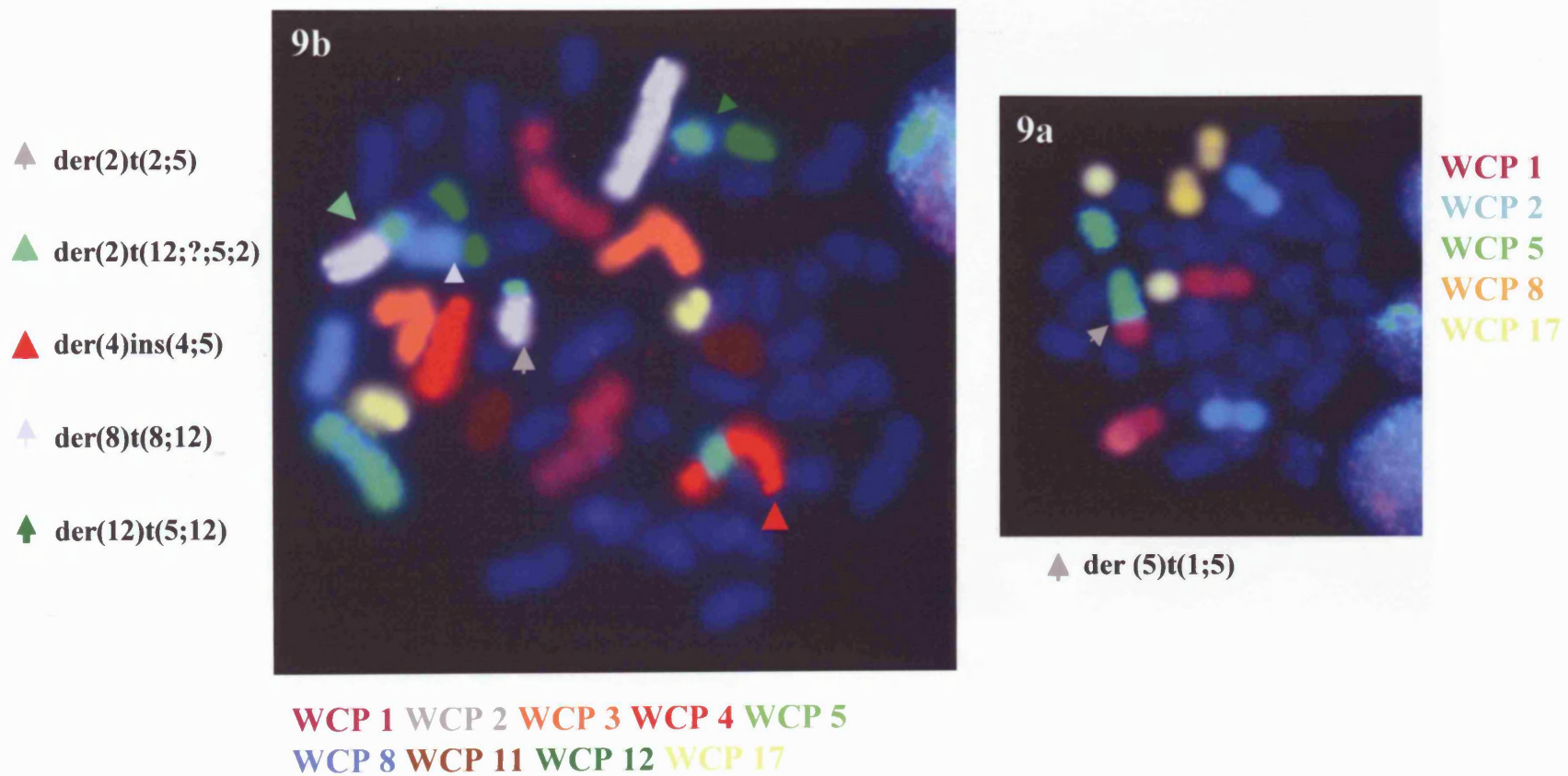
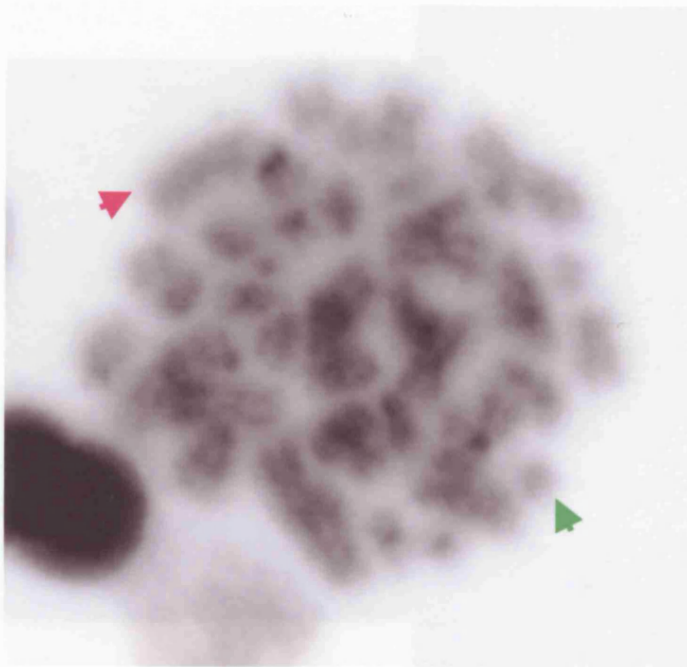
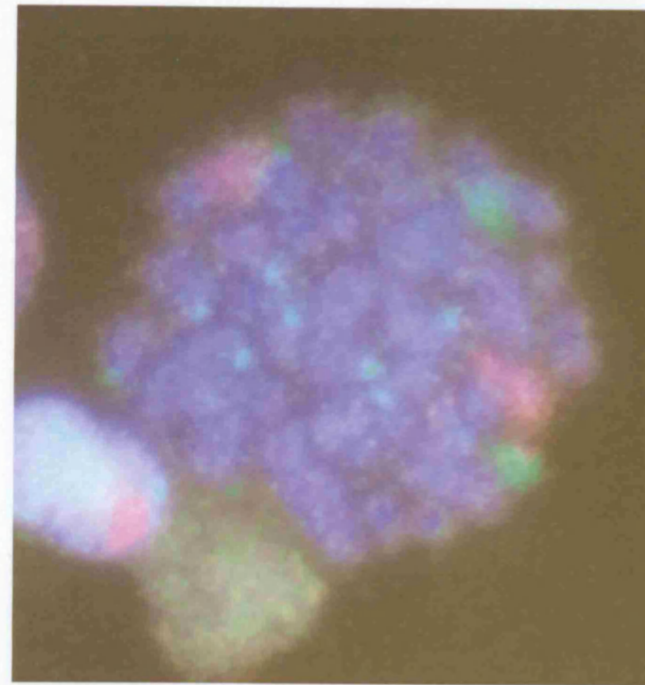


Fig V:9 Five markers detected by sequential FISH

(case 10 table V:1 UIN:33)



▲ mar der(?)t(?;12;21) ▲ der(21)t(12;21)



WCP12 WCP21

Fig V:10a Variant t(12;21)

(case 11 table V:1 UIN:100)

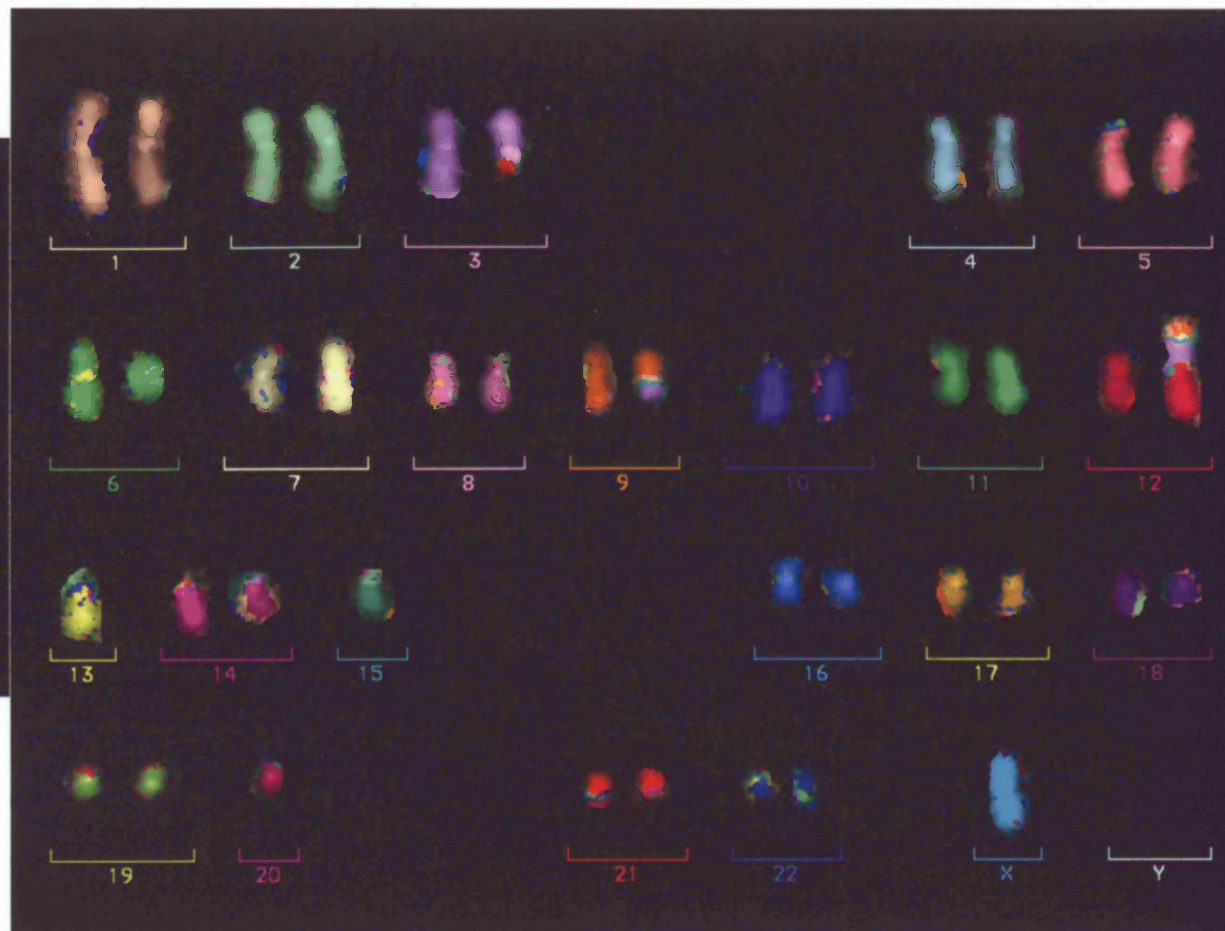
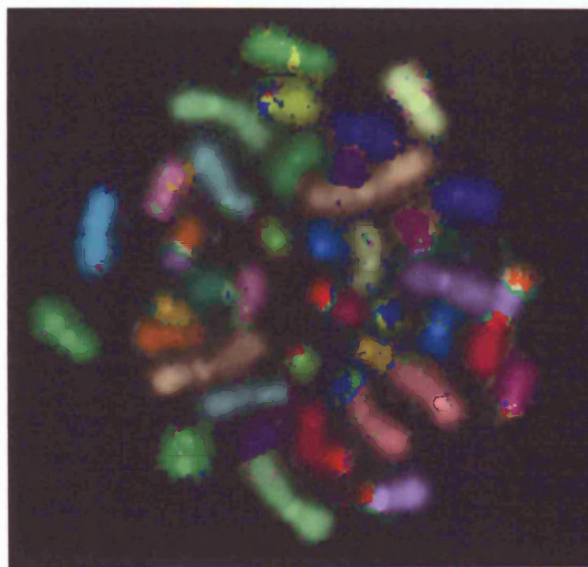
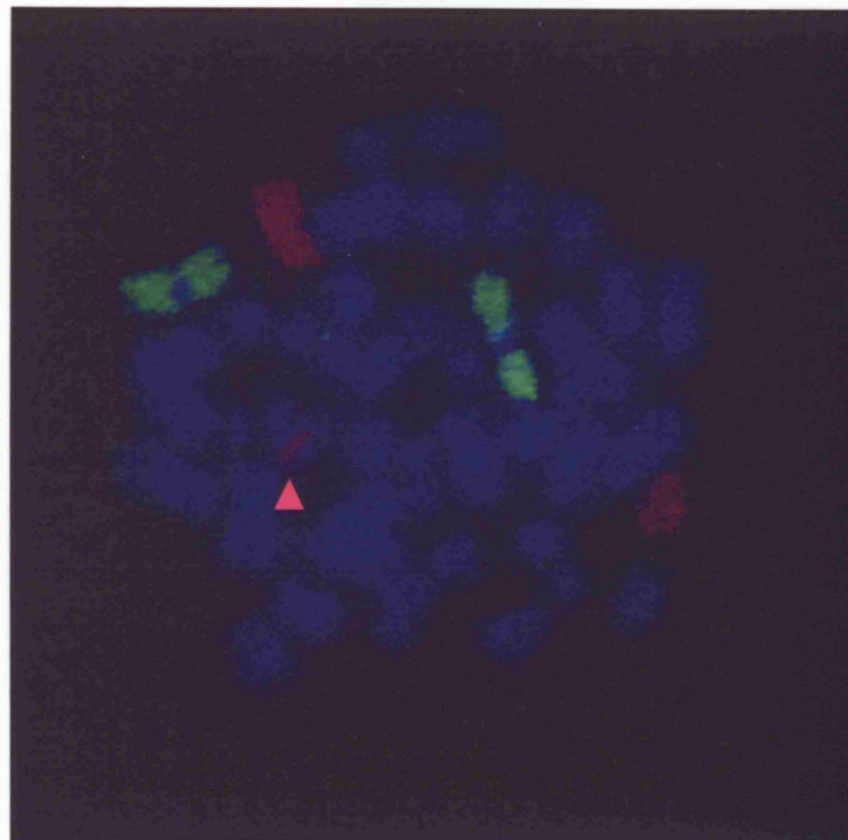


Fig V:10b Variant t(12;21) M-FISH

(case 11 table V:1 UIN:100)

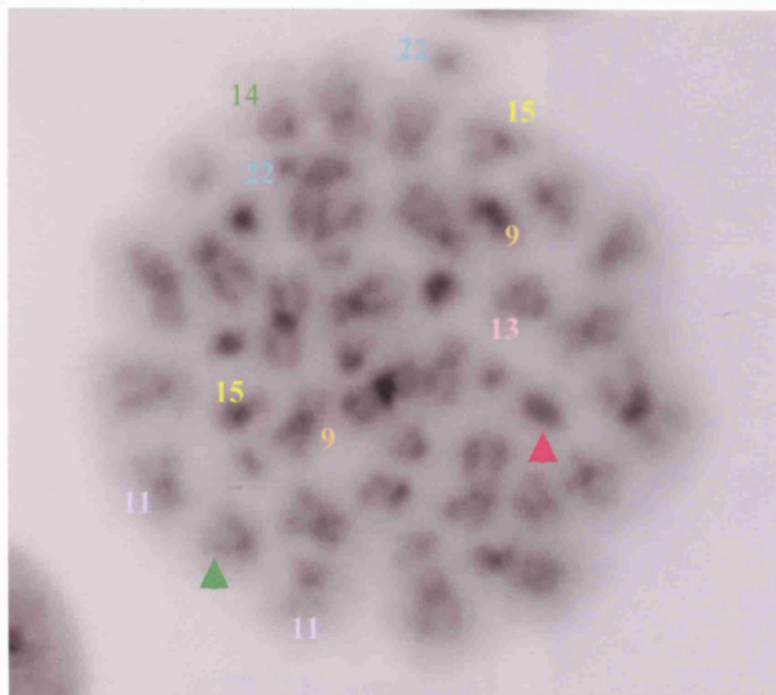
▲ der(?)ins(?;6)



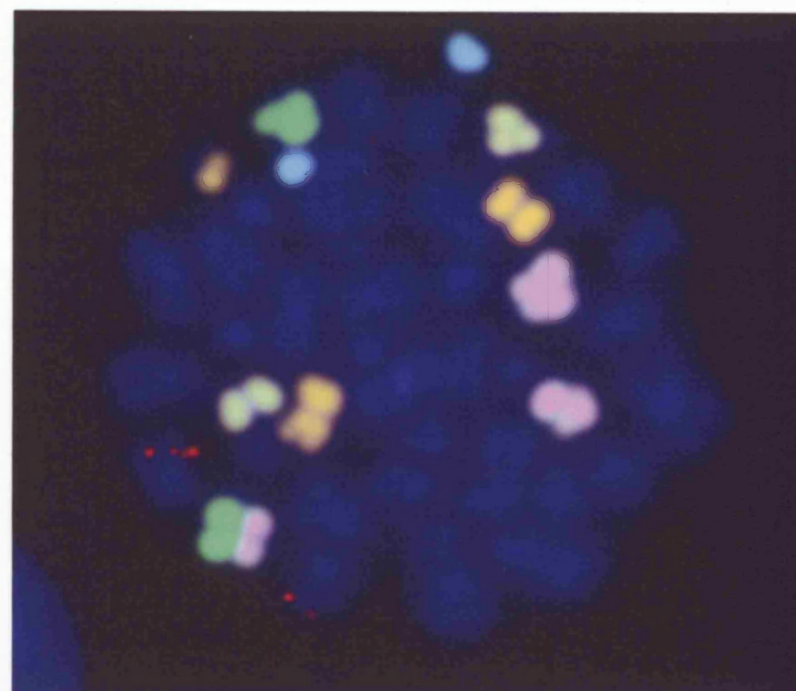
WCP1 WCP6

Fig V:11 Marker with der(?)ins(?;6)

(case 12 table V:1 UIN:119)



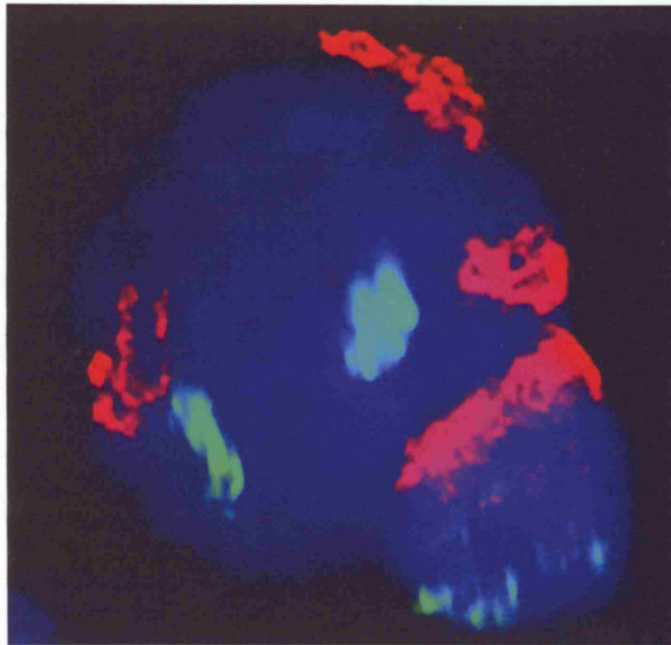
- ▲ del(13)
- ▲ ins(14;13)



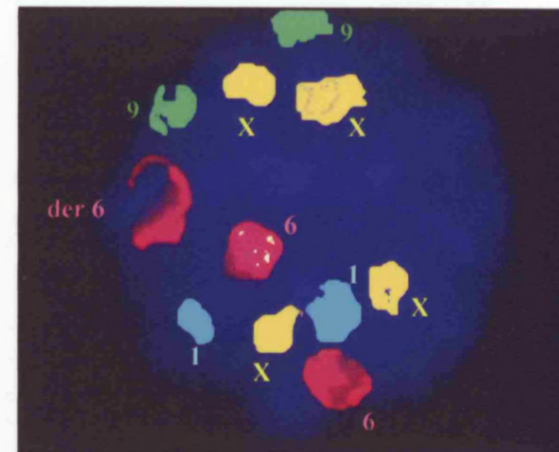
MLL WCP9 WCP13 WCP14 WCP15 WCP22

Fig V:12 ins(14;13) in *BCR/ABL* positive ALL

(case 13 table V:1 UIN:144)



WCP6 WCP12



WCP1 WCP6 WCP9 WCPX

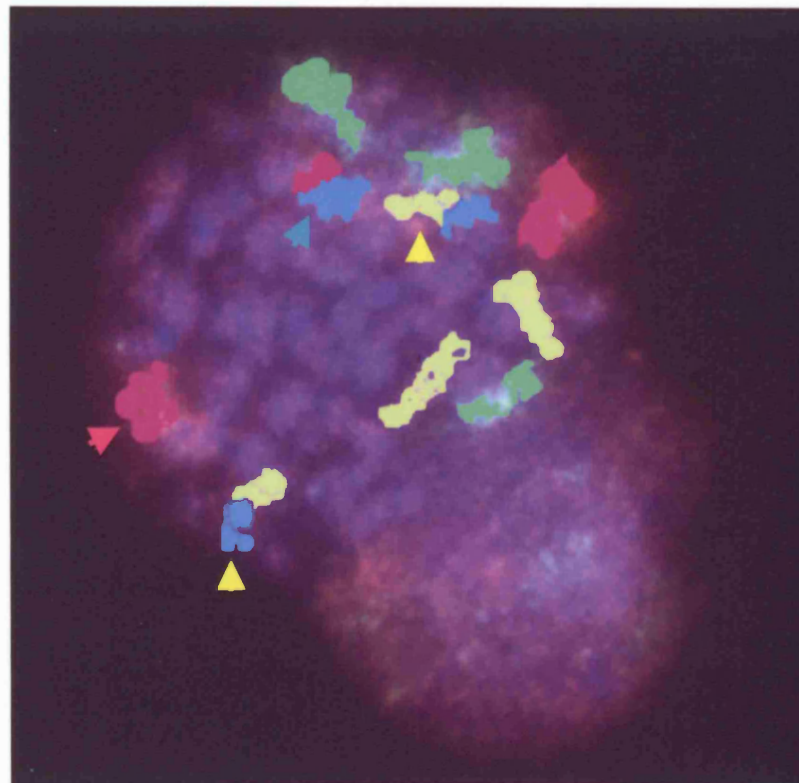
Fig V:14a Sequential Interphase FISH after failed cytogenetics

(case 15 table V:1 UIN:174)

▲ **der(X)t(X:13)**

▲ **der(6)t(?:6)**

▲ **der(13)t(6:13)**



WCPX WCP4 WCP6 WCP13

Fig V:14b Sequential Interphase FISH after failed cytogenetics

(case 15 table V:1 UIN:174)

Chapter VI

**Interphase FISH screening is useful in risk
assessment for adult patients with ALL**

VI: 1 Abstract

Multiplex interphase FISH for *MLL* gene rearrangements, *BCR/ABL* and *ETV6/AML1* fusions in addition to interphase FISH tests for hidden numerical chromosomal changes, was carried out on a series of adult patients with ALL. Complete follow up information was available for 159 patients, in whom the impact of cytogenetic abnormalities including additional information from FISH studies could be assessed in relation to outcome.

Twenty cases (12.4%) with t(9;22)(q11;q34) , six cases (3.7%) with translocations involving *MLL*, seven cases (4.4%) with t(12;21)(p13;q22), nine cases (5.6%) with other *ETV6* rearrangements and seven cases with hidden numerical chromosomal abnormalities were detected by FISH.

The detection of these abnormalities by cytogenetic studies and / or FISH ensured all patients were accurately identified and allowed prognostic classification of patients. Patients with t(9;22), *MLL* or *ETV6* rearrangements showed a poor prognosis when compared with patients with normal karyotypes. The only group with a good outcome detected in this study was high hyperdiploidy, in which no high-risk structural abnormalities were detected.

Using a simple combination of cytogenetics, FISH results, and presentation white cell counts we identified three prognostic groups differing in EFS and OS.

VI: 2 Introduction

ALL may be viewed as a spectrum of diseases, ranging from chemotherapy sensitive disease in which patients have long-term survival, to extremely resistant disease associated with short survival times.

With the use of intensive chemotherapy, it has become possible to identify several factors influencing treatment outcomes, including sex, age, immunophenotype, WBC count, response to induction treatment, cytogenetics at presentation and the detection of minimal residual disease at various points during treatment (Hoelzer 1988; Boucheix 1994; GFCH 1996; Secker-Walker 1997; Mortuza 2002). Another factor that may influence outcome is the use of high dose treatment with transplantation as consolidation chemotherapy, a question that is being addressed by the current MRC U.K. adult ALL treatment trial (UKALL XII). It is hoped that in the future our ability to distinguish risk groups at presentation will allow a more individualised approach to patient treatment.

Both numerical and structural chromosomal abnormalities are commonly detected in leukaemic blasts; and their presence allows the identification of groups with significantly different outcomes. In the previous adult ALL trial UKALLXa, from a review of 350 patients, 232 patients had abnormal clones. Using a hierarchical method for classification significantly poorer outcomes were seen in patients with t(9;22), t(4;11) or other 11q abnormalities, and hypodiploidy (<46 chromosomes). A somewhat better outcome was noted in patients with 12p abnormalities or hyperdiploidy (50-60 chromosomes) (Secker-Walker 1997). Similar results were seen in a French study of 443 adult ALL patients (GFCH 1996) confirming the poor

outcome associated with t(9;22) and 11q23 abnormalities. An additional group of patients with hypodiploidy (30-39 chromosomes) and associated triploidy, were identified as poor risk, while an improved outcome was seen in patients with near tetraploidy in addition to those with high hyperdiploidy. More recently, CALGB have published the findings from cytogenetic analysis of 256 patients who were diagnosed since 1984. The high risk of trisomy 8 and monosomy 7 were described as well as the possible good outcome seen in association with 12p abnormalities (Wetzler 1999); This is the only study presenting long term follow up of patients, over 10 years in some cases.

Due to the low incidence of many of the described chromosomal changes, firm conclusions on their influence on prognosis are difficult to reach. For example, the good outcomes observed in patients with 12p abnormalities from the UKALLXa study represented only 13/350 patients, similarly the good outcome associated with near tetraploidy was based on 9/443 patients. The poor outcome described in association with trisomy 8 and monosomy 7 was found in 23/256 and 14/256 patients respectively, only 11 and 5 of whom were Ph negative.

It is important in the design of risk-adapted treatment protocols in ALL that adequate cytogenetic information is available for all patients. However, due to difficulties of cytogenetic analysis in ALL; up to 25% of cases fail in large multi-centre series (Secker-Walker 1997; Wetzler 1999) a proportion of the chromosomal abnormalities may be cytogenetically undetectable (as they are present in a non-dividing population of cells or produce only subtle chromosomal change) molecular methods are increasingly being used to complement conventional cytogenetics

(Devaraj 1995; Scurto 1998).

The application of FISH allows chromosomal abnormalities to be identified in interphase and metaphase cells on a cell-by-cell basis. Using locus specific probes for gene rearrangements and chromosome specific alpha satellite centromeric probes (Romana 1993; Kasprzyk and Secker-Walker 1997) it is possible to rapidly assess large numbers of cells and identify patients with gene rearrangements or alteration in chromosome number, even in the absence of adequate metaphases for complete karyotypic analysis. Unlike RT-PCR with specific primers, the use of multiplex FISH will also detect: abnormalities arising from alternative breakpoints within the *BCR* and *ABL* or *ETV6* and *AML1* loci; other abnormalities involving these genes; and cases of hidden aneuploidy. With the availability of commercial FISH probes, it has become possible to undertake screening for the chromosomal abnormalities of prognostic significance which have influence on the predicted outcome in patients with ALL (Ritterbach 1998; Avet-Loiseau 1999).

This study evaluates interphase FISH for the detection of *BCR/ABL*, *ETV6/AML1* and *MLL* rearrangements, as well as aneuploidy and the impact of these abnormalities on outcome in adult ALL.

VI: 3 Materials and methods

VI: 3: 1 Patients

Diagnostic bone marrow (148 samples) and peripheral blood (11 samples) were available from 159 adults with ALL presenting between February 1993 and April 2000. Patient details are given in Chapter II. All patients were entered to the current UK Medical Research Council adult ALL treatment trial (UKALLXII). The criteria for entry are age ≥ 15 years; newly diagnosed ALL; excluding mature B-cell ALL.

The trial was designed to evaluate the efficacy of bone marrow transplantation or peripheral blood stem cell transplantation in the treatment of adult ALL. Patients with matched related donors receive an allogeneic transplant, while those without donors are randomly assigned to receive chemotherapy or an autologous transplant. In patients randomly allocated to receive chemotherapy, conventional maintenance therapy is given for 2 years. They also receive 24 Gy cranial irradiation. In a modification to the initial protocol, the trial is now assessing the effect of *ABL* tyrosine kinase inhibitor ST1 571 (Imatinib) on maintaining remission after chemotherapy as well as after any transplant procedure for patients with Ph positive ALL. Following allogeneic or autologous transplants patients receive no maintenance therapy, except Imatinib for Ph positive patients.

Although the trial continues to recruit, data up to May 2000 have been published (Durrant 2000). Patient data are shown in (Table VI.1).

Clinical details, treatment and outcome data were available for 159 patients within the UKALL XII trial and form the basis of this study. Of these patients 55 had undergone a transplant procedure in first complete remission. In 39 patients this was

allogeneic, while in 12 patients it was autologous. There were three matched unrelated donor (MUD) and one syngeneic transplant. A further five allogeneic transplants, five MUD and one autologous transplant were undertaken at relapse.

For FISH studies nine bone marrow samples were selected as controls from adult patients with normal karyotypes and non-malignant hematological disease, as previously described in Chapter II.I.

VI: 3: 2 Probes for FISH

Details of all probes used are presented in Chapter II:4:1).

Specific probes:

Commercial probes for *BCR/ABL*, *MLL* and *ETV6L/AML1* were used in interphase FISH studies.

Centromere specific probes:

Multiprobe®-I (Cytocell) or single indirectly labelled alpha satellite probes (Oncor) were used for chromosome number assessment.

VI: 3: 3 Methods of analysis

Details of cytogenetic analysis, immunophenotyping and equipment used for FISH analysis are presented in Chapter III: 2: 3.

FISH:

All probes were hybridized according to manufacturers' instructions, as detailed in Chapter II:5.

Interphase FISH

All cases were tested by interphase FISH for *BCR/ABL*, *MLL* and *ETV6/AML1*.

Cases with failed or normal cytogenetics results were also studied by the Multiprobe-I system for chromosome enumeration.

Statistical analysis:

Statistical analysis was undertaken using SPSS software.

Differences between chromosomal subgroups in terms of age and white blood count were tested by the chi-squared test.

Two end points were studied, the time to death from any cause (overall survival) and the time to relapse or death in remission (event free survival). Univariate analysis of several possible confounders on these end points were then examined using the Kaplan Meier and Log rank test. Confounders with significant influence were then entered in a multivariate cox regression model.

VI: 4 Results

Cytogenetic and FISH results

Samples from 159 patients were tested; an abnormal clone was detected in 100 cases, only normal metaphases were found in 34 cases. In the remaining 25 cases, conventional cytogenetics failed to produce a result. Interphase FISH screening for *BCR/ABL*, *MLL*, *ETV6/AML1* abnormalities was successful in all 159 patients. In 17 of those with a failed cytogenetic result screening for aneuploidy was also successful.

Hierarchical classification

The features of patients according to cytogenetic group, including those with established structural abnormalities (45 patients) or numerical abnormalities (38 patients) are shown in (Table VI:2).

Structural Abnormalities:

The subgroup of patients with Ph chromosome was the largest, having 20 cases (12.4%), three of which were only detected by FISH. Additional chromosomal abnormalities were seen in 13 cases, including a complex Ph translocation (2 cases), an additional Ph (2 cases), monosomy 7 (4 cases), or chromosomal gains resulting in low or high hyperdiploidy (one case each). Ph positive patients tended to be older (mean age 36y compared to 28y for the group as a whole) ($p=0.05$), but did not have higher WBC's (mean 17.4 compared to 9.7 for the group as a whole) ($p=0.72$), although the majority were of the pre-B/C-ALL immunophenotype the difference was not statistically significant.

Six patients had 11q23 abnormalities, including five with t(4;11) one with t(11;19).

Additional chromosomal abnormalities were present in four cases; high hyperdiploidy in two and i(7)(q10) in one. Patients with 11q23 abnormalities were more likely to have WBC $>100 \times 10^9/l$ ($p=0.0001$), and five of the six had a pre-B immunophenotype.

Seven patients had the cryptic t(12;21) detected by FISH, in four cases cytogenetics had failed and in the remaining three cases 12p abnormalities were found by conventional cytogenetics, with deletion 12p in one and additional material on 12p in two cases. The patients ranged in age from 15-50 years, all seven expressed the c-ALL phenotype.

Twelve patients showed 12p abnormalities without evidence of the *ETV6/AML* rearrangement. Abnormalities were detected by conventional cytogenetics in eight of these, deletions of 12p were present in three cases, translocations involving 12p were found in four cases, two of which showed the dicentric, dic(9;12)(p11;p11). In one case additional material was present on 12p. In a further four cases the presence of the abnormal 12p was only detected by FISH as loss of one (three cases) or both (one case) copies of *ETV6*, without cytogenetically visible deletions of chromosome arm, 12p. Patients with 12p abnormalities were younger ($p=0.01$).

Numerical abnormalities:

Nine patients were classified as hypodiploid, in which cytogenetics showed 45 chromosomes in seven cases and 38 chromosomes in one previously published case (Harrison 2004). Although cytogenetic analysis showed only 15 normal metaphases in one patient Multiprobe-I revealed an isolated monosomy 21 (case B table IV:1). In all but one case there were associated structural abnormalities, all but one

expressed the c-ALL phenotype.

Low hyperdiploidy, with chromosome counts of 47-49 was found in 15 patients, seven having 47 chromosomes by cytogenetic analysis. Three patients with failed cytogenetic results were shown by Multiprobe-I to have low hyperdiploidy, with isolated trisomy 21 in two cases (cases E and F, table IV:1) and trisomy 8 in the third (case C, table IV:1). Isolated trisomy 8 was found by Multiprobe-I in a further case with normal cytogenetics (case D, table IV:1). Additional structural abnormalities were present in seven cases. There was no association with age group, immunophenotype, or WBC.

Fourteen patients showed high hyperdiploidy, ten were younger than 20 years, all had WBC $<20 \times 10^9/l$ and in ten, blasts expressed a c-ALL phenotype.

A total of 42 patients were classified as normal (defined as normal karyotype, or failed conventional cytogenetics, and negative FISH for gene rearrangements and where possible for chromosomal abnormalities); 31 had normal cytogenetic results, eleven had failed cytogenetic results. Multiprobe-I testing was negative in seven of those with failed cytogenetics and 20 of those with normal karyotypes. These patients with normal results accounted for 46% of patients with T-ALL immunophenotype.

In view of the description of trisomy 8 as an isolated high risk factor (Wetzler 1999), patients with trisomy eight were examined separately. Among eleven patients with trisomy 8 it was the sole chromosomal abnormality in two cases, associated with low (three cases) or high (five cases) hyperdiploidy in eight cases. In two cases with normal and one with failed cytogenetics, trisomy 8 was detected by FISH screening

with Multiprobe-I. It was the sole abnormality in two cases and associated with high hyperdiploidy in one. In six cases the numerical abnormalities were associated with structural abnormalities, half of which were undefined marker chromosomes. Patients with trisomy 8 had WBC ranging from $2-20 \times 10^9/l$.

Influence of cytogenetic / FISH defined hierarchical classification on outcome:

The mean event-free survival for the 159 patients on UKALLLXII was 3.5 years (95% CI 2.9-4). Overall disease-free survival at three years was 45.6% and at five years was 33.5%.

Patient groups with structural or numerical chromosomal abnormalities were compared with the group of patients with normal cytogenetics and FISH results. Survival curves are shown in figures VI:1 and VI:2. The Ph positive patients showed unfavourable outcomes when compared to patients classified as normal ($p=0.03$ for EFS, and $p=0.01$ for OS). The patients with 11q23 abnormalities were difficult to assess due to small numbers.

Of interest, we found that in contrast to some previous studies, patients with 12p abnormalities showed a tendency to shorter event free survival ($p=0.05$). This result persisted when the seven patients with t(12;21) were added in the analysis ($p=0.08$). Despite the small numbers, the nine patients with FISH evidence of *ETV6* deletion showed statistically significant shortened event free survival ($p=0.01$).

Patients with hypodiploidy or low hyperdiploidy showed no significant difference in survival from the group as a whole.

Patients with high hyperdiploidy showed a trend for improved survival comparable

to that seen in the normal group

Patients with trisomy 8 had EFS and OS similar to those of the patient group as a whole, but shorter than the normal group.

Prognostic groups by combination of cytogenetics, FISH and WBC

Previously described factors with an impact on OS or EFS which influence death or freedom from relapse; in childhood and / or adult ALL were studied among our patient series, these included:

Male sex, WBC above $100 \times 10^9/l$,

B-lineage immunophenotype,

Slow early responders (time to complete remission of more than 28 days)

Cytogenetic and / or FISH evidence of the presence of the unfavourable abnormalities: t(9;22), 11q23 abnormalities, trisomy for chromosome 8, hypodiploidy, or the favourable markers: including normal cytogenetics, high hyperdiploidy, 12p abnormalities, t(12;21).

The effect of treatment received (i.e. the use of chemotherapy versus transplant procedure of any type in first remission) was also studied.

Tables VI:2 and VI:3 show results of univariate analyses of the above factors on end points studied. Variables adversely influencing outcome were found to be: age over 42 years, WBC over $100 \times 10^9/l$, cytogenetic / FISH finding of t(9;22), *MLL* rearrangement, or *ETV6* abnormalities. On the other hand, patients with normal cytogenetics / FISH or high hyperdiploidy showed improved outcome. Using a combination of cytogenetic findings, FISH and WBC it was possible to define three

distinct risk groups, allowing better definition of those groups with better outcome.

Survival curves are shown in figures VI:3 and VI:4.

High Risk group (n=35): Patients with t(9;22), *MLL* gene rearrangements, and *ETV6* abnormalities (excluding those with t(12;21))

Low risk group (n=52): Patients with high hyperdiploidy, normal cytogenetics / FISH; WBC<100x10⁹/l.

Intermediate risk group (n=72): Patients not classified into the other two groups.

The identification of these groups has led to a clear separation in the Kaplan Meier survival curves.

There is more than five fold difference in survival for the low risk compared to the high-risk group (Median survival and event free survival of 60 & 61 months compared to 11 & 13 months respectively, $p=0.003$ & $p=0.006$).

Multivariate analysis: For EFS, analysis of 159 cases identified age of >42 years as the most significant factor relating to poor outcome ($p=0.01$), after adjusting for age, the risk grouping system remained a significant factor ($p=0.02$), patient sex, disease immunophenotype, time to achieve complete remission and treatment protocol were not related to outcome.

For OS, analysis of 159 cases identified age of >42 years as the most significant factor related to poor outcome ($p=0.003$), after adjusting for age the risk grouping system remained a significant factor ($p=0.013$). Patient sex, disease immunophenotype, time to achieve remission and treatment protocol were not related to outcome.

VI: 5 Discussion

Over the decades a number of factors related to presenting features have been described in adult ALL that influence treatment outcome and survival. Immunophenotype, total WBC and time to achieve complete remission have all been found to affect prognosis. The strongest predictor of outcome however remains the presence of distinct chromosomal abnormalities at presentation.

A number of these factors are currently incorporated into treatment algorithms in childhood ALL, with more intensive therapy reserved for patients with high WBC, high risk cytogenetic features or failure to rapidly clear bone marrow of blast infiltration. The uniformly poor outcome in adults with ALL has thus far limited such an approach for this patient group.

The patients presented in this study are being treated within the ongoing UKALLXII trial; follow up time is variable in the majority of these patients, ranging from 1-8 years. Based on karyotypic information for 134 patients with follow up details, (Fig VI: 1&2) it was apparent that Ph positive patients continue to do poorly with event free survival rates of <20%, compared to 57% at three years in patients with normal karyotypes. However, the previously described improved outcome for patients with t(12;21) was not apparent in this group, with event free survival rates of <20% at 3 years. Similarly trisomy 8 did not appear to associate with poorer outcome (43% EFS at 3 years). A new finding in this study was the poor outcome found in the subgroup with *ETV6* deletions and 12p abnormalities, this is contrary to previous studies of childhood ALL (Rubnitz 1997). Patients with high hyperdiploidy and no high-risk structural abnormalities were the only group to do better than those with

normal cytogenetics, with survival rates exceeding 60% at 3 years.

It was clear from this study of 159 patients that the application of interphase FISH testing carried out as a routine screening approach was effective in the accurate detection of poor prognosis patients. This was demonstrated by the identification of three patients with hidden *BCR/ABL*, and nine patients with *ETV6* abnormalities. However it was equally effective in the detection of eight patient with good outcome identified from negative FISH results, despite failed cytogenetics.

In addition to the direct effect of chromosome abnormalities detected by conventional cytogenetics or FISH, we have shown that by using a simple ranking system dependent on cytogenetics / FISH and total WBC at presentation, it is possible to identify three subgroups with significantly different event-free and overall survival. The results were based on relatively small numbers, thus this risk grouping needs to be confirmed by application to large patient groups. It appears to hold promise due to its simplicity and the strength of its influence on outcome, exceeding that of individual factors, including time to remission. It may assist in treatment option decisions, particularly for the group of patients identified as low risk.

Cytogenetic analysis is routinely undertaken on all cases of ALL as a requirement for patients entered into clinical treatment trials. In both children and adults the cytogenetic results influence treatment choices, especially induction chemotherapy, and in adults, the option of allogeneic bone marrow or stem cell transplantation.

With the recent advances in modes of treatment targeting clone specific products, such as the *ABL* tyrosine kinase inhibitors ST1 571 (Imatinib) (Goldman 2000;

Ottmann and Hoelzer 2002; Wassmann 2002; Wassmann 2003), which has been used for the treatment of Ph positive ALL patients in UKALLXII since June 2002, it has become essential that every effort be made to provide complete cytogenetic information for all adults presenting with ALL.

The proven superior sensitivity of FISH confirms that this approach is the best technique to use as an adjunct to conventional cytogenetics to rapidly and accurately identify patients with high and low-risk abnormalities at the time of diagnosis. FISH provides speed and reliability in the detection not only of gene rearrangements, but also aneuploidy. Screening for *BCR/ABL* fusion was successful in 176/180 (99%) of samples tested in our laboratory. The success rates for *MLL* and *ETV6/AML1* were 99% and 97% respectively. The use of directly cultured bone marrow for the preparation of interphase nuclei or cytospin preparations (Horton 2000) means that FISH results can be available within 24 hours. The systematic use of FISH, as a screening tool for t(9;22)(q34;q11), t(12;21)(p13q22), *MLL* rearrangements and high hyperdiploidy in all adult patients with ALL, not only provides more accurate information on the incidences of these abnormalities, but helps to make more precise assessment of long term outcome in these patients.

In combination with cytogenetics and WBC, FISH screening may provide sufficient diagnostic information to allow the introduction of risk adjusted treatment strategies in adults similar to those already in place for children.

Table VI:1 Data from MRC UKALL XII Trial

Data as of May 2000	MRC
Registered	707
Early deaths (<84 days)	56
Early relapse	8
No CR but alive	8
Treated < 13 weeks	19
Allogeneic BMT	206
MUDT	40
Eligible for randomization	370
Randomized	161
Elected ABT	21
Elected chemotherapy	108
Unknown	80

CR: Complete remission BMT: Bone marrow transplant
MUDT: Matched unrelated donor transplant
ABT: Autologous bone marrow transplant

Table VI:2 Clinical outcome by cytogenetic groups

		t(9;22)		11q23		t(12;21)		Abn 12p		+8*		Ho (34-45)		HeL (47-49)		HeH (50-60)		Norm	
		N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Total	159	20	12.6	6	3.7	7	4.4	12	8	11	7	9	6	15	9	14	9	42	26
Sex																			
Male	95	11	12	4	4	5	5	7	7	5	5	6	6	9	9	10	11	25	26
Female	64	9	14	2	3	2	3	5	8	6	9	3	5	6	9	4	6	17	27
Age																			
15-19	38	0	0	1	3	3	8	0	0	6	16	2	5	5	13	10	26	7	18
20-29	45	8	18	1	2	2	4	8	18	1	2	1	2	3	7	0	0	13	29
30-39	27	3	11	2	7	0	0	0	0	1	4	1	4	2	7	1	4	10	37
40-49	36	8	22	1	3	1	3	3	8	1	3	4	11	4	11	1	3	9	25
50-55	13	1	8	1	8	1	8	1	8	2	15	1	8	1	8	2	15	3	23
WBC																			
<10	77	8	10	0	0	6	8	4	5	8	10	4	5	10	13	12	16	21	27
10-49	43	6	14	1	2	0	0	4	9	3	7	4	9	3	7	2	5	14	33
50-99	13	1	8	0	0	0	0	3	23	0	0	0	0	2	15	0	0	2	15
>100	26	4	15	5	19	1	4	1	4	0	0	1	4	0	0	0	0	5	19
Immno																			
C-ALL	73	10	14	0	0	7	10	3	4	7	10	8	11	7	10	10	14	17	23
Pre-B	33	3	9	5	15	0	0	5	15	3	9	0	0	2	6	2	6	10	3
Null	1			0	0	0	0					0	0					1	100
T-ALL	24			0	0	0	0	2	8			0	0	2	8			11	46
Mixed	1			0	0	0	0					1	100						
Other	6	1	17	1	17	0	0					0		2	33	1	17		
*EFS Med mo	26	13		6		28		12		24		27		26		45		58	
+EFS 3Y (%)	46	0		30		NA		28		41		37		NA		53		57	

*EFSMed mo: Median event free survival in months

NA: follow up less than 3 years

+EFS 3Y (%): (%)3-year event free survival

+8* these patients are also included in the HeL or HeH groups

Table VI:3 Effects of risk factors on event free and overall survival

Table 1. The Effects of Risk Factors on Event-Free and Overall Survival									
Event free survival						Overall survival			
	Category	No	Med mo	P	RR (95% CI)	No	Med mo	P	RR
Risk Group	Low	52	60	0.003	0.6 (0.3-1.2)	52	61	0.006	0.6 (0.3-1.2)
	Intermediate	72	26		1	71	36		1
	High	35	11		1.7 (0.9-3.34)	35	13		2.0 (1.0-4.2)
Age	<42 y	118	27	0.02	0.4 (0.2-0.8)	117	40	0.002	0.3 (0.2-0.7)
	>42 y	41	15		1	41	15		1
Sex	Male	95	24	0.42	1	95	34	0.66	1
	Female	64	60		0.9 (0.6-1.8)	63	35		0.9 (0.5-1.7)
WBC	<100 x10 ⁹ /l	134	28	0.008	1	133	47	0.004	1
	>100 x10 ⁹ /l	25	9		0.5 (0.1-2.0)	25	13		0.5 (0.2-0.9)
Immuno	B-type	106	24	0.06	1	106	34	0.27	1
	T-type	24	51		0.6 (1.6-2.9)	24	50		0.7 (0.2-2.8)
Time to CR	<28 days	67	28	0.15	0.7 (0.4-1.3)	66	38	0.55	1.0 (0.5-1.7)
	>28 days	63	20		1	63	34		1
Treatment	BMT	55	44	0.83	1.6 (0.9-2.9)	52	48	0.64	1.0 (0.5-1.8)
	Chemo	93	28		1	93	34		1
No: number		Med mo :median in months			BMT :bone marrow transplant				

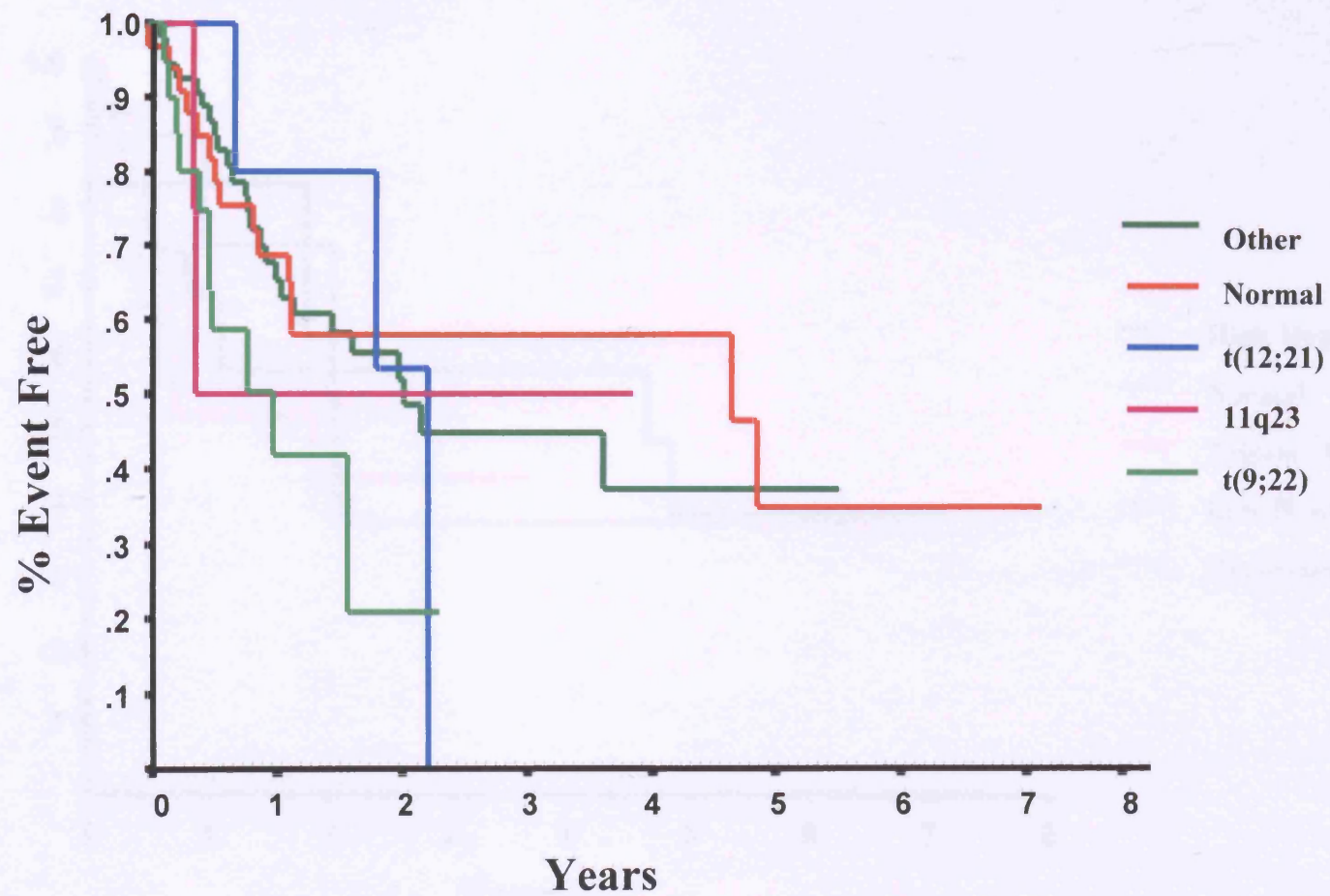


Fig VI:1 Event free survival in patients with structural abnormalities

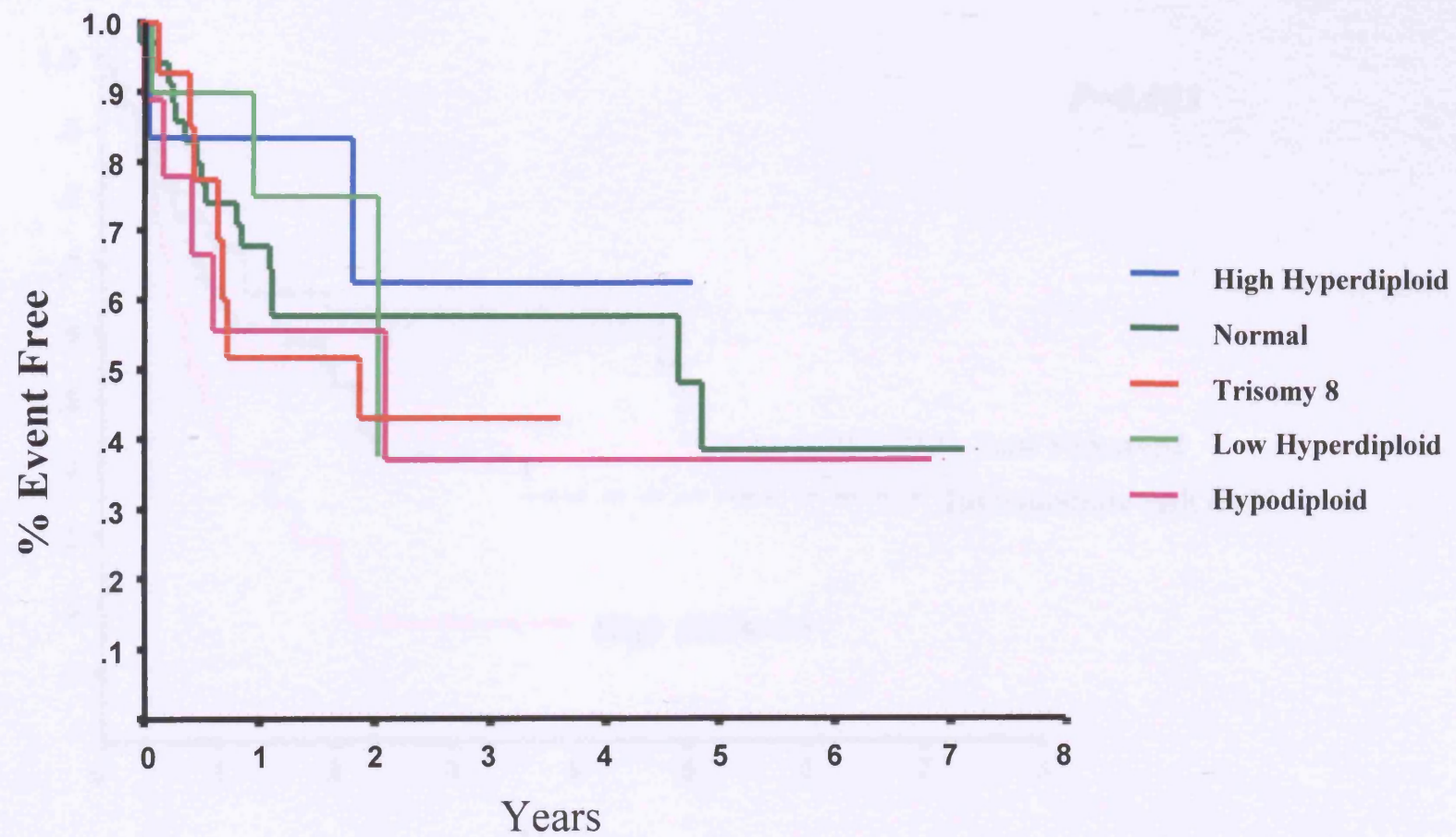


Fig VI:2 Event free survival in patients with numerical abnormalities

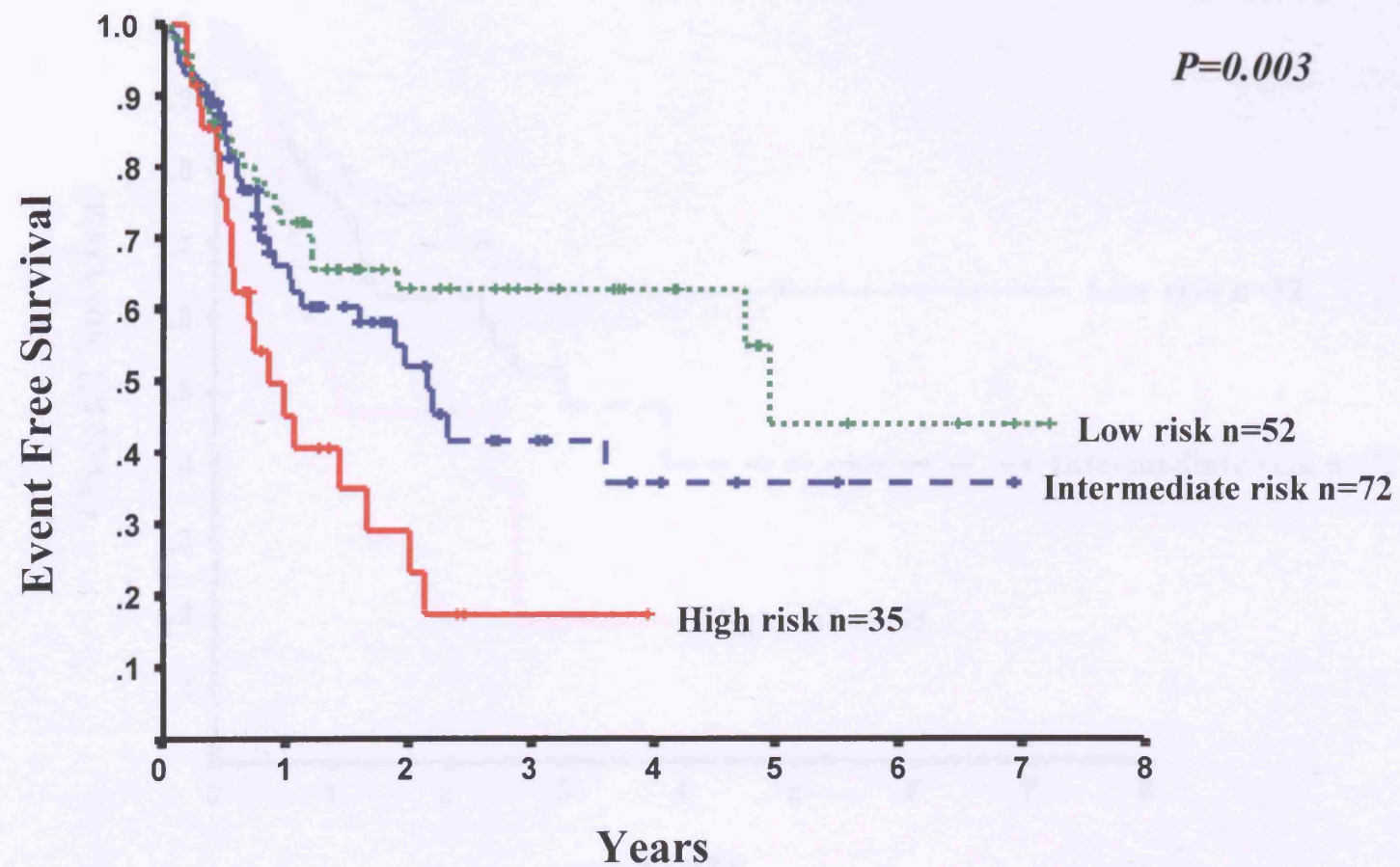


Fig VI:3 Event free survival by prognostic grouping

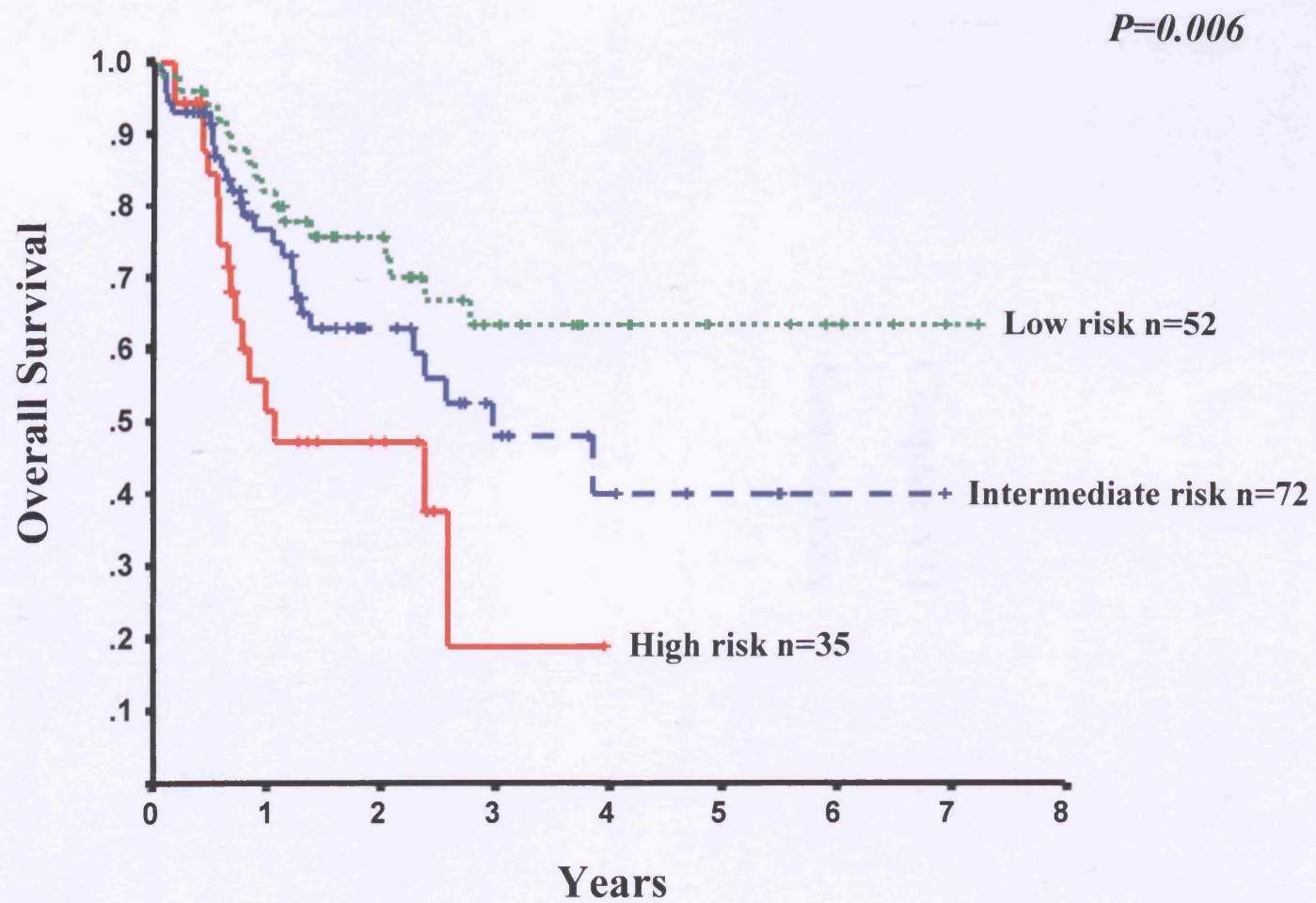


Fig VI:4 Overall survival by prognostic grouping

Chapter VII

Conclusions

VII: 1 FISH improves cytogenetic success rate in adult ALL

FISH analysis for the *BCR/ABL* fusion was successful in 176 (99%) cases and detected a Ph translocation in 23 cases (13%) of which six cases were not detected by cytogenetic analysis, as described in Chapter III. This demonstrates that these patients would not have been identified if cytogenetics alone had been relied on.

In sixteen of 153 (6.5%) Ph negative patients, gains of *BCR* and/or *ABL* signals were identified, in a number of cases this finding led to the identification of hidden numerical chromosomal abnormalities. Overall FISH detected alterations involving the *BCR* and or *ABL* genes in 22% of patients.

FISH with the *MLL* probe was successful in 174 cases, with an *MLL* rearrangement found in seven patients (4%). The *MLL* probe independently detected all cases of t(4;11)(q21;q23) seen by cytogenetic analysis, as well as the two cases with the cytogenetically subtle t(11;19)(q23;p13) one of which had been described as add(19). Two cases were identified with *MLL* rearrangements, which had been reported as deletions of 11q23. Although FISH did not detect any 11q23 abnormality that had not been seen by cytogenetics, the involvement of *MLL* was confirmed in all cases. These observations confirm the advantages of FISH in identifying abnormalities of 11q23 involving *MLL*, including translocations with all chromosomal partners, as well as *MLL* deletions. This may be significant in adults as the outcome in children with ALL with all *MLL* gene rearrangements is the same as that for patients with t(4;11).

Seven of 162 (4.3%) cases tested were found to be positive for the *ETV6/AML1*

fusion. This incidence in adults is much lower than that of 25% reported in childhood B-lineage ALL. A similar incidence was previously published from results of RT-PCR,(Aguiar 1996). Our series included one FISH positive case that was RT-PCR negative, suggesting that FISH provides a more accurate method of screening for this abnormality including detection of cases with variant breakpoints not seen in the routine RT-PCR process. Two of our positive cases were 45 and 50 years old respectively, representing the oldest *ETV6/AML1* fusion positive patients reported.

Outcome of adults with t(12;21) has not previously been reported. Our study, although only covering a short period of follow up suggested these were not dissimilar to overall poor outcome results for adults. Losses or gains of *ETV6* and/or *AML1* signals were identified in 31 of 155 (20%) negative patients. In total interphase FISH detected abnormalities involving the *ETV6* and or *AML1* genes in 23.5% of cases.

FISH using the Multiprobe-I for aneuploidy was successful in 48 cases, including 28 cases with a normal karyotype, and 20 with failed cytogenetics. A clone with a numerical chromosomal abnormality was detected in twelve (25%) cases.

High hyperdiploidy of >50 chromosomes was found in five cases. The hyperdiploid clone was associated with apparent losses of chromosome 6 and 13 in one case and of chromosome 7 in another. Further studies using wcp helped to elucidate complex chromosomal abnormalities in both cases. Low hyperdiploidy of 47-50 chromosomes was found in four cases, with isolated trisomy 21 and trisomy 8 in two cases each. A further two cases showed hypodiploidy of 45 chromosomes and one

case showed near triploidy >70 chromosomes. The overall incidence of numerical chromosomal abnormalities detected in this study was 30.5%. One third of these cases were only detected by the application of FISH.

Metaphase FISH, including sequential FISH, was utilised to complement cytogenetic analysis in the identification of marker chromosomes in 29 patients. Marker chromosomes were identified in 15 cases. The marker chromosome was simple in five cases and complex in 10 cases. Patients with highly complex karyotype had a shortened survival.

Overall the combination of FISH techniques in this study provided additional cytogenetic information in 36/176 (20%) cases. Alterations in karyotype were, as expected, seen in significantly more cases with a failed cytogenetic result 15/35 (43%), than those with a successful cytogenetic analysis 21/141(15%). The changes altered prognostic expectation in 23 cases, five with a Philadelphia chromosome, one with t(11;19) and nine with *ETV6* abnormalities. In this study patients with *ETV6* rearrangements fared very poorly. Of equal importance was the finding of normal FISH results among a group of eight patients with failed cytogenetics and a good outcome.

VII: 2 Results of FISH can distinguish groups with differing prognosis

The detection of chromosomal abnormalities by cytogenetic studies or by FISH allowed prognostic classification of patients. A poor prognosis was observed in patients with t(9;22), *MLL* or *ETV6* rearrangements as compared to patients with a normal result from karyotype and FISH studies together. The EFS rate was <20%, compared to 57% in the normal cases. The previously described good outcome of children with t(12;21) was not apparent in this group of adults with EFS of <20% at 3 years. A poor outcome was seen in the subgroup with *ETV6* deletions and 12p abnormalities, a finding contrary to previous studies in childhood ALL.

Patients with high hyperdiploidy without evidence of high-risk structural abnormalities continue to do well, with survival rates exceeding 60% at 3 years. Whereas patients with trisomy 8 do not appear to have a poorer outcome than those with normal karyotypes (43% EFS at 3 years), contrary to previous reports.

Using a combination of cytogenetics, FISH, and presentation white cell count we distinguished three distinct prognostic groups:

High Risk group

Unfavourable outcome comprised patients with t(9;22), *MLL* gene rearrangements, and *ETV6* abnormalities. These patients fared poorly ($p=0.003$, and $p=0.006$ for event free and overall survival, respectively)

Low risk group

Favourable outcome included patients with high hyperdiploidy, and those with a

normal result from cytogenetics and FISH with presentation $\text{WBC} < 100 \times 10^9/\text{l}$.

Intermediate risk group

Patients without the poor outcome cytogenetics, or normal cytogenetics and FISH, but with high WBC showed an intermediate outcome. They fared less well than the favourable group ($p=0.05$, and $p=0.02$ for EFS and survival, respectively).

During the follow up period of the study compared to the low risk group, the probability of an event occurring in the high-risk group was increased almost threefold (2.933 1.4-6.1 95% CI), for the intermediate risk group the probability was increased by a factor of 1.7 (0.87-3.22 95% CI).

When compared to the low risk group the likelihood of death was increased by over threefold (3.53 1.51-8.2 95% CI) in the high-risk group, with a doubling in the probability of death (2.3 1-4.75 95% CI) in the intermediate risk group.

VII: 3 FISH screening should be incorporated into the diagnostic workup for adults with ALL.

In the current and previous trials for the treatment of AML in the UK (AML 12 and AML 15), the role of cytogenetics was integral to the prognostic classification of patients at presentation and assisted in the choice of post remission induction chemotherapy. Patients within the good prognosis category being treated with high dose chemotherapy and stem cell / bone marrow transplantation only in second remission.

The results from this study suggest that a similar approach could be taken for adults with ALL. Using the simple combination of presentation WBC, cytogenetic and FISH results, a subgroup of low risk patients with considerably better overall outcomes was identified. For this subgroup treatment strategies that do not include high dose therapy in first remission should be considered. For this approach to be implemented, FISH screening for the structural abnormalities involving *BCR/ABL*, *MLL*, *ETV6/AML1* and screening for high hyperdiploidy must be incorporated to the diagnostic workup for these patients. We have shown that these tests can be undertaken rapidly and reliably using commercially available probes on small amounts of patient material, providing results within days of patient presentation. Thus clinicians can make rapid decisions regarding treatment options.

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